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Relationship between an inflammatory mucosal T cell response and susceptibility of sheep to *Teladorsagia circumcincta* infection

Virginia Mauro Venturina

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This humble piece of work is dedicated to
my mighty Creator,
and my family:
to my caring mother who will never see what she had dreamed me to become,
to my enduring father for the infinite love,
to Alberto for his love that knows no bounds,
and to my great bundle of joy and inspiration:
Aldrian Gabriel,
Job Rafael,
and Keziah Isabel

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Preface

‘‘*I treasure the great “ride on the bus” in my journey to the PhD diploma. There were crossroads, humps and bumps, and detour at some point but that just made the journey worthwhile. Otherwise, I could have just fallen asleep on the bus and missed the wonderful sights along the way and the entertaining chat with the people on board”.*

-Virginia

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The chapters on this thesis are a product of seemingly-endless mental exercise. The decision for me to take the opportunity to do a PhD has proved that it is not only about what I could get from that opportunity but what that opportunity can take away from me.

It was not easy and I hope that it is worth it.

Declaration of Originality

I hereby declare that the work presented in this thesis and the thesis itself have been composed and originated by myself, unless otherwise specified. No part of this work has been, or will be submitted for any other degree of professional qualification

Virginia Mauro Venturina

August 2012

The Roslin Institute

Royal (Dick) School of Veterinary Studies

Easter Bush, Midlothian

University of Edinburgh

EH25 9RG

List of Publications

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List of Abbreviations

A. General abbreviations

Δ	delta
°C	Degrees Celsius
µg	microgram
A	alanine
AAM	Alternatively activated macrophage
Ab	antibody
ABM	Abomasal mucosa
ALN	Abomasal lymph node
Amp	Ampicillin
ANOVA	Analysis of Variance
APC	Antigen presenting cell
AR	Anthelmintic resistance
ATP	Adenosine triphosphate
BLAST	Basic Local Alignment Search Tool
bp	base pairs
BSA	Bovine serum albumin
CD	Crohn's Disease
CD	Cluster of differentiation
cDNA	complementary DNA
cDNA	Complementary deoxyribonucleic acid
Ch	Chromosome
CIP	Calf intestinal phosphatase
CMI	Cell-mediated immunity
Cq	Quantifiable cycle
Ct	Threshold cycle
dATP	Deoxyadenosine triphosphate
DC	Dendritic cell
dCTP	Deoxycytidine triphosphate

dGTP	Deoxyguanosine triphosphate
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
dNTP	Deoxynucleotide triphosphate
DTT	Dithiothreitol
dTTP	Deoxythymidine triphosphate
dUTP	Deoxyuridine triphosphate
E/S	excretions/secretions
EB	Elution buffer
EDN	Eosinophil-derived neurotoxin
EDTA	Ethylenediamenetetracetic acid
ELISA	Enzyme linked immunosorbent assay
FACS	Fluorescence-activated cell sorting
FEC	Faecal egg count
g	Gram
G	Guanine
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GC	Guanine or cytosine
gDNA	Genomic deoxyribonucleic acid
GI	Gastrointestinal
GSP	Gene-specific primers
H&E	Hematoxylin and eosin
HCl	Hydrochloric acid
HPRT	Hypoxanthine-phosphoribosyltransferase
HRM	High resolution melt analysis
IBD	Inflammatory bowel disease
IEC	Intestinal epithelial cell
IPTG	Isopropyl β-D-1-thiogalactopyranoside
LB	Luria-Bertani broth
LPS	Lipopolysaccharide

M	Molar
$MgCl_2$	Magnesium chloride
$MgSO_4$	Magnesium phosphate
MHC	Major histocompatibility complex
min	Minute (s)
ml	Millilitre
mM	Millimolar
MMC	Mucosal mast cell
mRNA	Messenger ribonucleic acid
MS	Multiple sclerosis
NaOH	Sodium hydroxide
ND	Not done
ng	Nanogram
NK	Natural killer
nm	Nanomole
NO	Nitric oxide
NTC	Non-template control
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
pDNA	Plasmid deoxyribonucleic acid
PI	Post infection
QTL	Quantitative trait loci
RFLP	Restriction fragment length polymorphism
RIN	Ribonucleic acid integrity number
RLM-RACE	RNA Ligase-mediated Rapid amplification of cDNA ends
RNA	Ribonucleic acid
RT	Reverse transcriptase/transcription
RT-qPCR	Reverse transcriptase- quantitative polymerase chain reaction
SD	Standard deviation

SDS	Sodium dodecyl sulphate
sec	second (s)
SMI	Sequence manipulation suite
SNP	Single nucleotide polymorphism
SYBR	Synergy Brands
T	Thymine
TAP	Tobacco acid pyrophosphatase
Taq	*Thermophilus aquaticus*
TH1	T Helper 1
TH17	T Helper 17
TH2	T Helper 2
Tm	Melting temperature
Treg	Regulatory T cell
TSLP	Thymic stromal lymphopoietin
UTR	Untranslated region
UV	Ultra-violet
X-Gal	5-bromo-4-chloro-3-indolyl- beta-D-galactopyranoside

B. Abbreviations for gene symbols

Gene symbol	Gene name	Gene symbol (where different)
EBI-3	Epstein_Barr virus induced 3	EBI3
FOXP3	Forkhead box P3	
GAPDH	Glyceraldehyde 3 phosphate dehydrogenase	
GATA 3	GATA binding protein 3	GATA3
IFNγ	Interferon gamma	IFNG
IgA	Immunoglobulin A	IGHA1
IgE	Immunoglobulin E	IGHE
IgG	Immunoglobulin G	IGHG
IgM	Immunoglobulin M	IGHM
IL-10	Interleukin 10	IL10
IL-12p40	Interleukin 12 p40	IL12B
IL-17A	Interleukin 17A	
IL-2	Interleukin 2	IL2
IL-4	Interleukin 4	IL4
IL-5	Interleukin 5	IL5
IL-6	Interleukin 6	IL6
IL-7	Interleukin 7	IL7
IL-7R	Interleukin 7 receptor	IL7R
IL-21	Interleukin 21	IL21
IL-21R	Interleukin 21 receptor	IL23A
IL-22	Interleukin 22	IL22
IL-23A	Interleukin 23A	IL23A
IL-27p28	Interleukin 27p28	IL27p28
J chain	J chain	IGJ
PIGR	Secretory component / poly Ig receptor	
RORγt	Retinoic acid receptor-related orphan gamma t	

RORA	Retinoic acid receptor-related orphan receptor A	
RORC	Retinoic acid receptor-related orphan receptor C	
SDHA	Succinate dehydrogenase complex subunit A	
SOCS	Suppressor of cytokine signalling proteins	
STAT1	Signal transducer and activator of transcription 1	
STAT2	Signal transducer and activator of transcription 2	
STAT3	Signal transducer and activator of transcription 3	
STAT4	Signal transducer and activator of transcription 4	
STAT5	Signal transducer and activator of transcription 5	
STAT6	Signal transducer and activator of transcription 6	
TGFβ	Transforming growth factor, beta 1	TGFB1
TLR	Toll-like receptor	
TNFα	Tumour necrosis factor alpha	TNF
YWHAZ	tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta	

Abstract

Control strategies against the parasitic nematode *Teladorsagia circumcincta* are problematic under current sheep management systems. Infection with the parasite, particularly in young lambs, results in significant production losses therefore sustainable worm control is being sought. It has been established that variation in resistance to *T. circumcincta* is under genetic control and the development of resistance is an acquired characteristic and has an immunological basis. This project investigated the immunological response to infection, of lambs with predicted resistance or susceptibility to *T. circumcincta*. Specifically, the study aimed to identify immune response-associated genes that were differentially-expressed in resistant and susceptible lambs and attempted to identify mutations in these genes. This study was part of a long term project that aims to identify genetic marker/s to aid in marker-assisted selection (MAS) for resistance to *T. circumcincta*.

Real time reverse transcription-quantitative polymerase chain reaction (real time RT-qPCR) was performed on abomasal mucosa and lymph nodes from 55 lambs used in a previous experiment. The lambs had been either trickle-infected with 2,300 infective larvae every two days over three months (infected resistant/susceptible, n=45) or sham-dosed (non-infected control, n=10). Lambs were ranked in relation to faecal egg count (FEC) and adult worm count (AWC) at post mortem; zero or low FEC (resistant) to high FEC (susceptible). Histopathology showed only mild pathological changes in the abomasal mucosa of resistant lambs but heavy lymphocytic inflammatory infiltration in the mucosa and submucosa of infected susceptible animals. Measurements of a range of cytokine transcripts and cell

markers associated with the four major CD4+ T cell subsets identified IL6, IL21, and IL23A as significantly increased by at least two-fold in abomasal lymph nodes and abomasal mucosa of susceptible lambs in comparison to resistant animals. Highly significant ($P<0.02$) positive correlations were found between IL6 ($\rho=0.35$), IL21 ($\rho=0.54$) and IL23A ($\rho=0.38$) transcript levels and AWC. Similarly, there were highly significant ($P<0.01$) positive correlations between FEC and IL6 ($\rho=0.41$), IL21 ($\rho=0.65$) and IL23A ($\rho=0.31$). In contrast, significant negative correlation ($P<0.04$) between IL23A with IgA antibody levels ($\rho=-0.31$) was found. There was also a significant positive correlation ($P<0.03$) of TGFB1 levels with AWC ($\rho=0.42$) and FEC ($\rho=0.32$) in the abomasal mucosa.

These data suggests that susceptibility to *T. circumcincta* is linked to the activation of the inflammatory TH17 T cell subset and that this chronic inflammatory response was inappropriate to clear worm infection. High resolution melt analysis failed to identify single nucleotide polymorphisms in the coding regions of IL21 and IL21R.

This is the first report of the involvement of TH17 response in GI worm infection in sheep. Similar gene expression studies involving the known upstream and downstream players of the TH17 response could be done.

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Keywords: Sheep; Nematode; *Teladorsagia circumcincta*; susceptibility; TH17 cells; cytokines; RT-qPCR

Chapter 1 Introduction

1.1 Gastrointestinal (GI) parasitism

Gastrointestinal parasite infection is a major burden to the small ruminant industry. Higher demand for meat and diminishing pasture lands has led farmers to increase their stocking density (Taylor, 2012) whilst employing tactical approach in worm control with chemical anthelmintics. The consequent scenario is higher risk of infection especially in susceptible animals and ineffective worm control measures.

Sheep are infected with several species of GI nematodes but generally the most important species in terms of prevalence, pathogenicity, and economic importance are *Haemonchus contortus, Trichostrongylus colubriformis*, and *Teladorsagia circumcincta.* *Haemonchus* and *Trichostrongylus* species are prevalent in warm, Mediterranean and sub-tropical and tropical regions while *T. circumcincta* is found in cool, temperate areas (Craig *et al.*, 2006; Larsen and Anderson, 2009; Taylor, 2012; Wilson *et al.*, 2008). Adult *T. colubriformis* reside in the small intestine while *H. contortus* and *T. circumcincta* are both found in the abomasum. All three species undergo a typical direct life cycle (Figure 1.1) with no tissue migration apart from the temporary lodgement of the pre-adult stages in the GI mucosa. *H. contortus* feeds voraciously on blood while *T. colubriformis* and *T. circumcincta* nourishes on cellular secretions (Soulsby and Mönnig, 1982; Urquhart, 1996).

The economic impact of GI nematode parasites in sheep is related to increased anthelmintic treatments, increased labour for sheep management and production losses in terms of decreased live weight, and reduced survival in lambs (McLeod, 1995; Newton and Meeusen, 2003). Mortality is not uncommon particularly in young, susceptible lambs. However, morbidity is high and the associated pathology

results in significant production losses in meat, milk and wool (Taylor *et al.*, 2007; Urquhart, 1996). Continuous reinfection with ubiquitous GI nematode parasites and their high rate of establishment can reduce weight gain b up to 50% (Sargison, 2008). GI parasitism also causes losses associated with repeated treatments and reduced animal performance valued annually at £84M in the UK (Nieuwhof and Bishop, 2005) and $1.5 billion in the US (Newton and Meeusen, 2003).

1.2 Teladorsagia circumcincta

Teladorsagia circumcincta (previously known as *Ostertagia circumcincta*) is the most common GI nematode parasite in sheep in cool temperate areas. The adult worm, which measures 7–12 mm, lodges in the lumen of the abomasal mucosa and feeds on mucosal tissues and cellular secretions (Dunn, 1978; Soulsby and Mönnig, 1982).

1.2.1 Life cycle

T. circumcincta follows a direct life cycle (Figure 1.1) with no tissue migratory stage, which is typical of most strongyle[1] species (Soulsby and Mönnig, 1982). Their pre-parasitic developmental stages have greater resistance to desiccation and lower temperature. Temperature of 4–10°C is required for the eggs to hatch in 48 hours (McKenna, 1998; Jasmer *et al*., 1986). Development from L1 to L3 could be as short as two weeks at 11°C or could extend up to 10 weeks at 7°C (Gibson and Everett, 1972). However, high temperature of 30°C is still favourable for their development (Salih and Grainger, 1982). The higher tolerance of *T. circumcincta* to lower temperature enables them to survive over winter. The pre-infective larval

[1] Strongyle – nematode worms of the Order Stronglylida

stages feed on microorganisms in the faeces on soil until they reach the third stage infective larva (L3). The optimal faecal moisture content for *T. circumcincta* to develop through to L3 is 60% (Rossanigo and Gruner, 1995). Infection is acquired by ingestion of the infective L3 by grazing sheep. Early fourth stage larvae may cease development (hypobiosis[2]) and remain inactive in tissues as a result of unfavourable environmental conditions or host immune response (Soulsby and Mönnig, 1982; Urquhart, 1987). Larvae penetrate the abomasal gastric glands in 2–3 days after ingestion and develop into pre-adult stages (L4 and L5) before they mature into sexually active adults 17–21 days post-infection. The rate by which larvae establish through to the adult stage ranges from 3–50% (Aumont *et al.*, 2003).

[2] Hypobiosis – cessation of development of nematode larvae in the gut of the definitive host

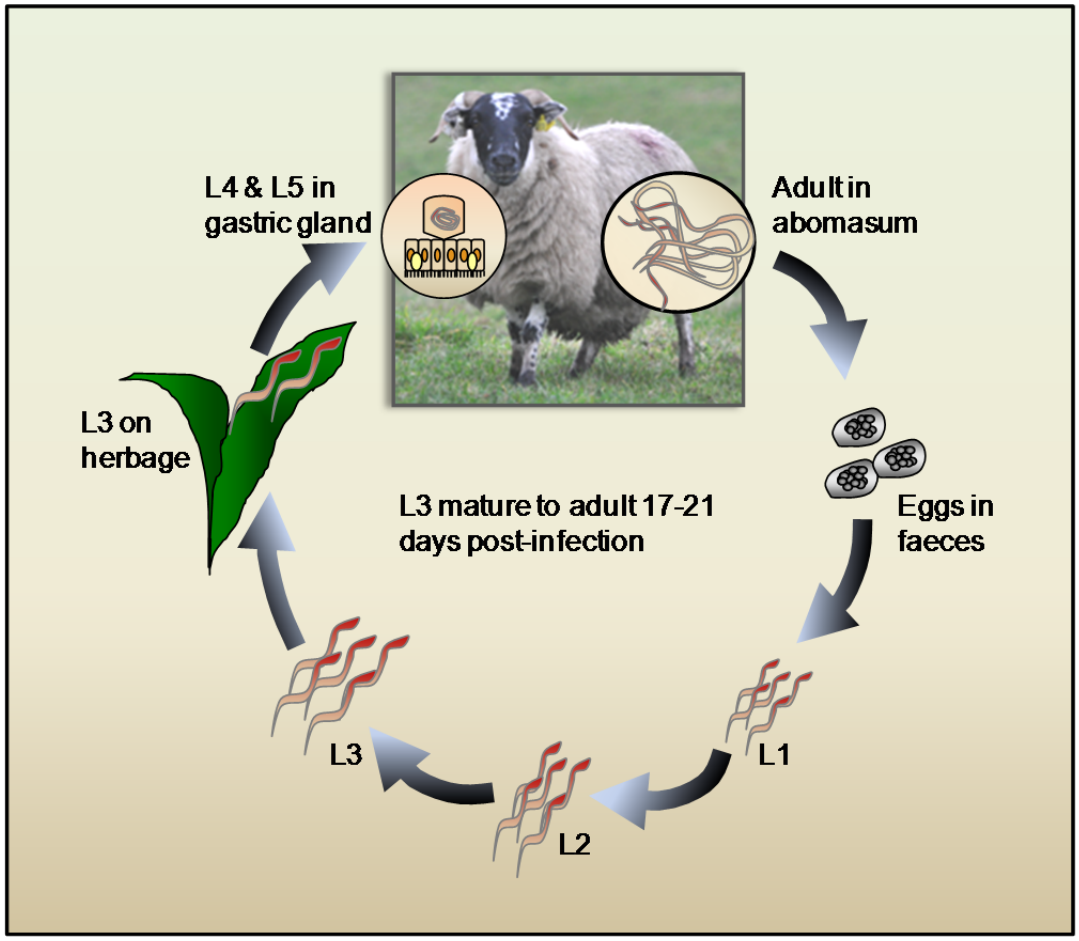


Figure 1.1 Life cycle of *Teladorsagia circumcincta.* Adults lodge in the abomasal mucosa and lay eggs, which are passed out with the faeces. Eggs hatch into the first larval stage (L1) within 1-2 days, then moult and develop onto L2 in 2–3 days. These stages feed on microorganisms in the faeces while they are trapped in the faecal pellet until they reach the infective larval stage (L3). L3 develops from 7 days to 3 months depending on the prevailing environment and may remain viable for several months. They climb up on blades of grasses to be ingested by ruminant host. The L3 enters the gastric glands where they moult again to become L4 then L5. They remain in the glands for 2–3 days and emerge into the lumen as adults.

1.2.2 Pathogenesis and clinical signs

T. circumcincta is a ubiquitous, persistent parasite that is responsible for seasonal outbreaks of parasitic gastroenteritis in weaned lambs (Gruner *et al.*, 1994; Taylor *et al.*, 2007; Urquhart, 1996; Sargison, 2008). The pathogenesis and associated clinical effects of *T. circumcincta* are attributed largely to the host immunologic response to clear infection (Houdijk *et al.*, 2003; Jackson *et al.*, 2009; Kyriazakis and Houdijk, 2006). Surveillance on the seasonal patterns of *T. circumcincta* infection suggests that fewer larvae survive the winter at pasture (van Dijk *et al.*, 2008). However, large numbers of ingested infective larvae undergo hypobiosis and remain as early L4 during winter and develop only to adult stages in spring (Sargison *et al.*, 2007; Waller *et al.*, 2004a). Hence, there is markedly reduced infection early in the year particularly for weaned lambs in their first grazing season. This pattern allows highly susceptible lambs to cause high build-up of pasture contamination later in the year increasing the risk of reinfection. High level of infection in spring may also be attributed to periparturient rise in faecal egg counts[3] by ewes.

The primary pathology of *T. circumcincta* infection is associated with larvae developing within the gastric glands. The host response to the invading L3 damages the tight junctions and this stretches the mucosal lining leading to hyperplastic abomasal mucosa (Gruner *et al.*, 1994; McKellar, 1993). Formation of nodules associated with larval development may be evident along with mucous cell hyperplasia. (Miller and Horohov, 2006; Scott *et al.*, 1998). These changes result in reduced or no production of digestive enzymes and leakage of macromolecules and

[3] Periparturient rise (PPR) in faecal egg counts or "Spring rise" is an increase in the number of nematode eggs in the faeces around parturition which is associated with relaxation of immunity resulting in more fecund females and resumed development of hypobiotic larvae (Urquhart, 1996).

proteins across the damaged mucosa resulting in hypoproteinemia, hypoalbuminemia and increased plasma pepsinogen (Lawton *et al.*, 1996; Simpson *et al.*, 2009). The disease is characterised by anorexia, intermittent diarrhoea, dehydration and weakness which may lead to emaciation and death if left untreated (Aitken, 2007; Kyriazakis *et al.*, 1998; Stear *et al.*, 2003; Sargison *et al*., 2008).

1.3 Development of anthelmintic resistance (AR)

The use of chemical anthelmintics for common GI nematodes of sheep has been the preferred method of worm control due to availability, cost, convenience and high efficacy. However, for the past 40 years resistance to one or multiple classes of anthelmintics, notably benzimidazoles, levamisoles, and ivermectin have been reported worldwide, and the incidence of resistance rising steadily (Gopal *et al.*, 1999;J ackson *et al.*, 2009; Kaplan, 2004; Waller *et al.*, 1995). In the UK, several reports of AR in sheep (Bartley *et al.*, 2003; Burgess *et al.*, 2012; Taylor and Hunt, 1989; Wilson and Sargison, 2007) have been recorded following its first account in 1982 (Britt, 1982). Several isolates of *T. circumcincta* have been shown to have multiple AR (Bartley *et al.*, 2004; Martinez-Valladares *et al.*, 2011; Sargison *et al.*, 2001; Sargison *et al.*, 2010; Sargison *et al.*, 2007; Sutherland *et al.*, 2008; Traversa *et al.*, 2007) such that even combination drenches were found to be ineffective (Wrigley *et al.*, 2006). The above discussion suggests that the use of chemical anthelmintics may not be a sustainable method of worm control.

It takes longer to discover, test and commercialize a new chemical anthelmintic than it is to maintain its efficacy. It was not until 20 years after the discovery of Ivermectin that a new class of synthetic anthelmintic amino-acetonitrile derivatives

(AADs) was introduced (Kaminsky *et al.*, 2008). Resistance to anthelmintics for sheep helminths may develop as early as three years after the initial drug use (Kaplan, 2004). It is therefore imminent that resistance to new drugs may develop after some time and integration of judicious anthelmintic use with non-chemical based worm control may be a sustainable approach.

1.4 Alternative strategies in worm control

The increasing worldwide problem of AR in sheep nematodes stimulates the development of non-chemical and sustainable methods of worm control. Work on these approaches has been the subject of several reviews (Hoste and Torres-Acosta, 2011; Jackson *et al.*, 2009; Molento, 2007; Stear *et al.*, 2006b) that are aimed at reducing worm infection by limiting host-parasite contact, enhancing the host immune response to reduce worm establishment and persistence, and removing adult worms in the host.

Protective immunity to GI nematode infections can be acquired after some time with repeated infections (Seaton *et al.*, 1989). Hence, it seems practical to employ management interventions targeted at boosting the immunological status of animals to prevent or reduce the effects of worm infection. There are three current strategies being tested to manipulate the immunological response of sheep to GI infections: nutrition boosting, vaccination and selection for resistance[4] and/or resilience[5].

[4] Resistance – ability to suppress the establishment and/or subsequent development of a parasite infection Bisset, S. A. & Morris, C. A. 1996. Feasibility and implications of breeding sheep for resilience to nematode challenge. *Int J Parasitol,* 26**,** 857-868.

1.4.1 Improved nutrition

High level of nutrition optimizes animal productivity and also the immune response to parasites. Parasitized lambs become weak and grow slowly because the nutrients required for their growth are reallocated to sustain energy required in the complex effector and immune-regulatory response mechanisms in parasite infection (Houdijk *et al.*, 2003; Jackson *et al.*, 2009; Kyriazakis and Houdijk, 2006). Indeed, lambs with continued milk supply improved resistance and resilience to experimental *T. circumcincta* infection compared to their twin weaned counterparts (Iposu *et al.*, 2008). This suggests that improved nutrition is an effective strategy to reduce the debilitating effects of parasite infection. While no cost analysis has been made to determine the actual benefits of improved nutrition enhancing resistance/resilience to worm infection, this strategy has high potential if adopted in combination with other options.

1.4.2 Pasture and grazing management

The basic concept of adopting grazing schemes for worm control is to reduce or prevent host contact with the infective stages of the parasite (Barger, 1999). Rotational grazing involves movement of animals from one paddock to another considering the supposed non-viability of L3 (Burke *et al.*, 2009); infective L3 larvae remain viable on pasture for up to a year in warm, humid areas (Barger *et al.*, 1994). This grazing method seems to work well in tropical (Barger *et al.*, 1994) but not so in sub-tropical and temperate regions (Eysker *et al.*, 2005) with decreased larval

[5] Resilience – the ability to maintain relatively un-depressed production while subjected to parasite challenge. Ibid.

challenge resulting in reduced worm load (Colvin *et al.*, 2012). Moving lambs to clean pastures is a practical anthelminthic treatment (Githigia *et al.*, 2001). However, there are practical implications with this approach, like provision of sufficient fodder and grass with limited area available for grazing and the initial cost of setting up the paddock divisions. Another scheme is to isolate the high egg shedders *i.e.* animals with high faecal egg count (FEC) from the flock, reducing worm burdens on the pasture.

Other interventions include the use of nematophagous fungi (Chandrawathani *et al.*, 2004; Waghorn *et al.*, 2002), surface-feeding earthworms (d'Alexis *et al.*, 2009) and physico-chemical agents (Bang *et al.*, 1990; Torres-Acosta and Hoste, 2008) on the pasture to prevent the development of larvae through the infective L3 stage. Aside from reducing host contact to the infective larval stage, boosting the host's ability to limit infections or cope with the harmful effects of parasite infection is another strategy for worm control (Hoste and Torres-Acosta, 2011; Knox *et al.*, 2012).

1.4.3 Development of vaccines

The development of vaccines including its strategic implementation has been a recognized immunological approach in worm control; however effective vaccines for GI nematodes remain elusive. To date, the only vaccine against a commercially-relevant ruminant nematode parasite is against the bovine lungworm, *Dictyocaulus viviparus,* where irradiated larvae give clinically effective protection. Studies on vaccine candidates for GI parasites have focused on directing immune responses toward possible targets on somatic, or secreted parasite molecules (Knox *et al.*, 2003; Smith and Zarlenga, 2006).

Less work has been done on vaccine development for *T. circumcincta* than with *H. contortus*. Why gut antigens protect against blood-feeding *Haemonchus* and mucosal browser *Ostertagia*, but not for *Teladorsagia* (Morton *et al.*, 1995) is not yet known (Smith, 2008). The immunogenic component of *T. circumcincta* has not been identified but candidate antigens have been located on the larval cuticle surface (Nisbet *et al.*, 2009), and in excretions/secretions (E/S) mixture (Nisbet *et al.*, 2011; Redmond *et al.*, 2006). However, vaccine trials using larval extracts (Halliday and Smith, 2011) and membrane glycoproteins (Smith *et al.*, 2001) have failed to establish effective protection against *T. circumcincta.*

Attempts have also been made to develop molecular vaccines, with recombinant versions such as H11 and H-gal-GP candidates for *H. contortus* but have failed to reproduce protection (Newton and Meeusen, 2003; Smith *et al.*, 2009a). Promising results on the potential use of nucleic acid vaccines has been demonstrated in *H. contortus* (Muleke *et al.*, 2007; Zhao *et al.*, 2012) but this work is still at a preliminary stage.

1.4.4 Breeding for resistance

Resistance is defined as the ability of a host to initiate and maintain responses to suppress the establishment of parasites and /or eliminate the parasite load (Woolaston and Baker, 1996). Utilizing genetic variation to select for sheep that are resistant to nematode parasites has been the subject of several reviews covering related work in the last three decades (Albers *et al.*, 1987; Bishop and Morris, 2007; Davies *et al.*, 2005; Gray, 1997; Stear *et al.*, 2009; Woolaston and Baker, 1996). To develop breeds that are selected for resistance to GI parasites seems to be the most promising

of all alternative worm control method. Improved resistance to nematodes translates to reduced costs incurred for anthelmintic treatments and diminished production losses attributed to worm infection. The breeding for resistance programme which primarily involved the use of phenotypic marker has been adopted successfully in Australia and New Zealand (Davies *et al.*, 2006; Gray, 1997; Windon, 1996). The major setback in the sustainability of its implementation is that some production traits are compromised in favor of low worm load. Development of phenotype markers to select for resistance to worms requires at least 6-7 years, not to mention the maintenance cost involved in keeping the animals.

1.4.4.1 Phenotypic traits as indicators of GI resistance

Selection for resistance has traditionally been based on quantitative measurements of phenotypic traits. The practical use of indicator traits for important GI nematodes is best exemplified by the FAMACHA scoring system. This is a scheme to assess the degree of anaemia as clinical manifestation of *H. contortus* infection in small ruminants. The method involves comparing the colour of the eye conjunctiva against an eye colour chart (Gauly *et al.*, 2004; Kaplan *et al.*, 2004), then marking a score that corresponds to the need to administer anthelmintic to the affected animal. Through this method, sheep resistant/resilient to *H. contortus* infection can be identified. Evaluation of estimated breeding values for FAMACHA scores indicate its heritability (Riley and Van Wyk, 2009) and has now been used as a phenotypic marker for selection in some farms in Brazil (Molento, 2007).

Most sheep breeding programmes for reduced egg production performed in New Zealand and Australia use FEC as the parameter for selection of resistance to GI

parasites (Windon, 1996;Woolaston, 1992). FEC has been the most widely-used parameter in identifying nematode resistance (Davies *et al.*, 2005; Gill, 1991; Gruner *et al.*, 2004; Smith *et al.*, 1984). Heritability values vary from 0.30 – 0.48 in *T. circumcincta* (Stear *et al.*, 2009), *T. colubriformis* (Douch *et al.*, 1996; Sreter *et al.*, 1994) and *H. contortus* (Gruner *et al.*, 2004) making it a viable indicator trait for selection. Its genetic component is further evidenced by its association with three different genotypes at the diallelic adenosine deaminase locus (Gulland *et al.*, 1993) and Major histocompatibility complex (MHC)-linked microsatellites in a wild population of Soay sheep (Beraldi *et al.*, 2007). Moreover, FEC is repeatable over time and heritable by six months (Bishop *et al*., 1996; Davies *et al*., 2005).

There are other potential phenotypic indicator traits in addition to FEC that could be selected for breeding sheep for resistance to nematodes. These include parasitological, immunological, and pathological characteristics (Beh and Maddox, 1996; Dominik, 2005). Examples are adult worm count and worm length of *T. circumcincta* with reported heritability of 0.14 and 0.62 respectively (Stear and Bishop, 1999). Plasma immunoglobulin A (IgA) is an immunological trait that has been found to have high heritability and repeatability (Strain *et al.*, 2002). Furthermore, some studies have claimed that eosinophil levels are useful, with estimated heritability in 4–5 month old lambs of 0.43–0.48 (Henderson and Stear, 2006). However, the reliability of this trait is debatable as high variability was observed in Australian sheep breeds (Douch *et al.*, 1996).

There are several challenges in the identification and development of phenotypic marker for resistance. The process involves carrying out *in vivo* studies under

condition when exposure and intensity of infection are critical to obtain statistically robust associations. In addition, environmental factors and physiological factors such as age, season and health status influence phenotypic markers (Jackson *et al.*, 2009; Stear *et al.*, 2007). Hence, phenotypic index of trait heritability warrants careful assessment and interpretation.

1.4.4.2 Development of genetic markers for resistance

Identification of molecular markers is a more reliable approach in selection for resistance or against susceptibility when direct association between the gene and the trait is determined. The genetic approach in breeding for resistance to worms involves identification of gene(s) that are linked to the trait to be selected. Genetic markers can only be defined with prior association to a quantitative trait such as FEC (Douch *et al.*, 1996). There are two general approaches in identifying causal mutation effect for quantitative traits: quantitative trait locus (QTL) mapping and candidate gene analysis (Carta and Scala, 2004; Pemberton *et al.*, 2012). The main differences between the two methods are described in Figure 1.2. Each has its own advantages and disadvantages and adoption depends on specific aims and available resources.

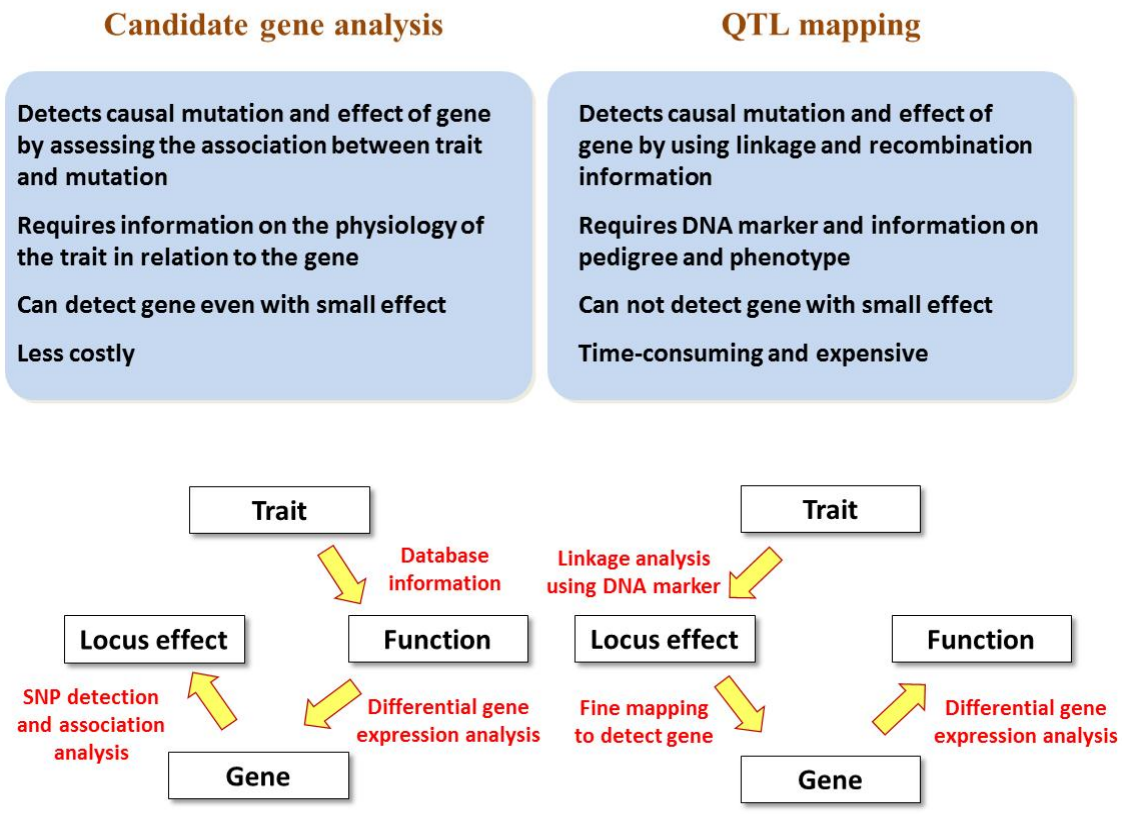


Figure 1.2 Approaches in identifying DNA marker for quantitative traits. The basic features of candidate gene analysis and quantitative trait loci (QTL) mapping are presented highlighting the advantages and disadvantages of both approaches. The work flow in detecting the gene that could be associated with a particular trait and the tools required in the process is shown.

1.4.4.2.1 Quantitative trait locus (QTL) mapping

QTL identification involves mapping a gene for a trait on genome region with the use of known DNA markers such as a microsatellite marker or single nucleotide polymorphism (SNP) (Beh *et al.*, 2002). SNP is a variation in the DNA sequence occurring when a single nucleotide in the genome (or other shared sequence) differs between members of a biological species. Microsatellite markers are repeating sequences of 2–6 base pairs of DNA which vary in number in any individual. The causal mutation associated with phenotypic traits found in that region is termed as QTL. The association between phenotypic value and known DNA markers is investigated to detect a QTL by using recombination[6] or linkage disequilibrium[7] information. The direct effect of QTL on an objective trait is then investigated by comparing to a DNA database of genes with known functions or gene expression analysis.

The traditional strategy of QTL mapping was to use linkage analysis to map a QTL with microsatellite marker. However, microsatellite markers are not as abundant as SNPs in the genome hence low marker density genome scans are generated yielding crude estimates of QTL location and magnitude (Slate *et al.*, 2009).

QTLs related to GI worm resistance have been identified at the genome- or chromosome-wide level. Regions of the ovine genome in Ch 3 was consistently associated with resistance to sheep strongyles (Beraldi *et al.*, 2007; Davies *et al.*, 2006; Dominik *et al.*, 2010; Marshall *et al.*, 2009). Loci in Ch 4 (Matika *et al.*,

[6] Recombination - the breaking and rejoining of DNA strands to form new molecules of DNA encoding a novel set of genetic information.
[7] Linkage disequilibrium - non-random association of alleles at two or more loci, that may or may not be on the same chromosome.

2011) and 6 (Beh *et al.*, 2002; Beraldi *et al.*, 2007) were also linked to worm resistance. Probably the most relevant QTLs associated with worm resistance, which may also be related to T cell differentiation, are those found in Ch 1 and 11 (Coppieters *et al.*, 2009; Marshall *et al.*, 2009). RORC (RORγ) and STAT3, transcription factors of TH17 cells, are both on Ch 1 and 11. Likewise, TBX21 and STAT5 transcription factors for TH1 and TH2 respectively are both located in Ch 11. In addition, Ch 20, which contains MHC Class II, has been suggested to influence resistance to strongyle worms (Coppieters *et al.*, 2009; Davies *et al.*, 2006). QTLs controlling a particular trait may hypothetically be unique for one disease or shared among different diseases. Since production of IgA may be stimulated by TH2 cells, and there is previous evidence that such cells promote resistance to *T. circumcincta*, it is possible that the phenotype of resistance/susceptibility is controlled by genes regulating TH differentiation.

Recently, genetic maps of sheep have become available covering most genomic regions (van der Werf *et al.*, 2007) and a large panel of SNP in sheep was made available to search for QTL by genome-wide association studies (Goddard & Hayes 2009). Ovine SNP chips that offers sufficient SNP density allows identification of over 50,000 SNPs in a single platform (Hayes *et al.*, 2012; Johnston *et al.*, 2011; Kijas *et al.*, 2009). This high-throughput genotyping technology gives hope to finding more QTLs for complex traits in a genome-wide association study. However, the disadvantage of this approach is that a very large animal/population sample is required to gain meaningful data making this approach very expensive.

1.4.4.2.2 Candidate gene approach

Candidate gene analysis evaluates a relationship between a trait and a mutation in specific functional genes selected for the differential expression in a particular phenotypic trait. These candidate genes are selected based on their established or supposed function using causal association tests. This method can be very powerful as it can detect trait loci even with very small effects. However, it requires a true candidate gene, meaning that a valid correlation between its expression and phenotypic trait of interest has been established. In this study, the immune-physiological response is measured and correlated with expression of genes associated with the immune response, i.e. cytokines and markers. Nucleotide polymorphisms are identified in differentially expressed genes, which will then be used as a basis in selecting candidate genes for genetic variation analysis.

1.4.4.3 Genetic association of resistance/susceptibility to GI nematodes

A number of genes have been linked with the ability of sheep to resist infection to GI parasites primarily based on FEC. Three molecular markers that are linked to GI nematode resistance phenotype have been identified: MHC-DRB1 which lies on Ch 20 (Buitkamp *et al.*, 1996; Sayers *et al.*, 2005a; Schwaiger *et al.*, 1995) the first intron of IFNG on Ch 3 (Coltman *et al.*, 2001b; Davies *et al.*, 2006; Matika *et al.*, 2011), and IL-4 on Ch 5 (Benavides, 2009b). A recent genome wide-scan of the African Red Maasai sheep has revealed novel QTLs associated with resistance to *H. contortus* and *T. colubriformis* located at Ch 6, 14 and 22 (Silva *et al.*, 2012).

Association of MHC genes with resistance or susceptibility to nematode parasites may be attributed to a high degree of polymorphism and involvement in the

induction and regulation of the immune response (Cresswell, 1994b). More specifically in *T. circumcincta* infections, protective response involves IgA activity (Halliday *et al.*, 2007; Stear *et al.*, 2004) and hypersensitive reaction (Greer *et al.*, 2008; Jackson *et al.*, 2004) to reduce worm burdens. Both mechanisms require a T cell response that entails antigen presentation through MHC Class II.

In different sheep breeds, significant associations between FEC reductions were reported in GI nematode species under natural and induced infections with other MHC class II alleles including *OLA-DRB 257* (Paterson *et al.*, 1998), and *Ovar-DY* (Buitkamp *et al.*, 1996). Susceptibility was also associated with the MHC Class II locus wherein high FEC was observed in New Zealand sheep carrying *OVAR DQA2*1201* allele (Hickford *et al.*, 2011).

One of the well-studied genetic markers associated with resistance to GI nematode is the allele *DRB1*1101* (also known as G2 and *Ovar-DRB1*0203*) of the MHC-DRB1 locus (Sayers *et al.*, 2005a; Schwaiger *et al.*, 1995). Studies have focused on the relationship of this locus with the physiological and immunological processes involved in the development of resistance to GI nematodes of sheep. Substitution of the most common allele by *DRB1*1101* has resulted in 22- to 81-fold reduction in *T. circumcincta* FEC in lambs (Sayers *et al.*, 2005a; Schwaiger *et al.*, 1995). Carrier lambs of *DRB1*1101* allele had lower adult worm burden and higher mast cell and plasma lymphocyte count (Hassan *et al.*, 2011). However, no difference in FEC was observed between carriers and non-carriers in this study suggesting that the resistance conferred is not dependent on fecundity, a parameter which was

consistently shown to mediate resistance in *T. circumcincta* infections (Halliday *et al.*, 2007; Smith, 2007).

Aside from MHC genes, a number of studies have identified mutations in cytokine genes associated with resistance. Low FEC and high *T. circumcincta*-specific IgA in free-living 4-month old lambs were associated with an allele at a microsatellite locus in the first intron of IFNG (Coltman *et al.*, 2001a). In addition, haplotype of the first intron of IFNG was found to be associated with low FEC in Texel and Suffolk breeds (Matika *et al.*, 2011; Sayers *et al.*, 2005b). A combined pathway analysis of QTL and gene expression information has confirmed IFNG and MHC II to be associated with immune functions and cell responses on resistance to internal parasites (Sayre and Harris, 2012). On the contrary, recent findings showed very little evidence linking IFNG with FEC (Dervishi *et al.*, 2011; Silva *et al.*, 2012), which is consistent with the work of (Beraldi *et al.*, 2007).

IL-4 has also been linked with reduced FEC when the most frequent allele was substituted by either IL-4*A or IL-4*C (Benavides *et al.*, 2009). Another allele, CSRD2138 (Benavides *et al.*, 2002) which lies close to IL-4 gene in Ch 5 has been associated with reductions in FEC after natural predominantly *H. contortus* challenge (Maddox *et al.*, 2001).

The importance of the association of IFNγ and IL-4 alleles to resistance to GI nematodes rests on both genes significance to T helper (TH) cell differentiation. The expression of IL-4 and IFNγ is critical to TH2 and TH1 type of immune response respectively (Mosmann *et al.*, 1986).

1.5 Immune response to GI nematode parasites

Protective immunity to GI nematodes is acquired after gradual, continuous, low-level exposure with infective larvae over time (Balic *et al.*, 2000b; MacDonald *et al.*, 2002; Miller, 1984). Protection is possible in primary infection but commences later compared to pre-infected lambs (Lacroux *et al.*, 2006). The greatest susceptibility to *T. circumcincta* infection occurs with weaned lambs during their first grazing season (Stear *et al.*, 1999). However, many sheep eventually control worm development and egg production. The cellular features of the immunological response in relation to the development of protection includes mucosal mastocytosis, increased numbers of globule leucocytes, and eosinophilia (Balic *et al.*, 2000a; Gill *et al.*, 2000; Schallig, 2000; Stevenson *et al.*, 1994; Urban *et al.*, 1991). How each of these response components contributes to protection is still unclear. The effector mechanisms of protection is signalled to a large extent by cytokines that leads to reduction or clearance of worm load characterized by worm expulsion, diminished worm length, reduced worm fecundity, and failure or delayed larval development (Finkelman *et al.*, 1991; Onah and Nawa, 2000).

Development of protective immunity against GI nematode infections primarily involves antibody response which is largely T-cell dependent. It is widely viewed that the response operates through the generation of parasite specific IgA and IgE antibody (Smith *et al*., 1986; Stear *et al*., 1999; Barger *et al.*, 1985; Halliday *et al.*, 2010), which acts to exclude larval colonization and minimize tissue-damaging inflammation (Macpherson *et al.*, 2000). Figure 1.3 summarizes the mechanisms of the existing paradigm on the immune response to infection with *T. circumcincta* and other GI worm parasites.

1.5.1 Activation of immune response to GI nematode infection

1.5.1.1 Signal induction of protective immune response

Cells of the innate immune system have been shown to promote signalling of TH2 response. Two Dendritic cell (DC) subsets have been identified in sheep: DC2 which was associated with TH2 cell type as it expresses high levels of IL-10 and DC1 which was linked to TH1 immune response owing to its up-regulation of IL-12p40 (Matthews *et al.*, 2007). Intestinal epithelial cells (IEC) are a major source of TH2 activating cytokines, notably thymic stromal lymphopoietin (TSLP), and IL-33 (Allakhverdi *et al.*, 2007; Owyang *et al.*, 2006; Schmitz *et al.*, 2005). Mast cells and eosinophils are known to initiate and maintain TH2 responses through their production of elevated levels of TH2 type cytokines IL-4 and IL-13 (Gessner *et al.*, 2005). Eosinophils may also act as an antigen presenting cell for the induction of the primary and expansion of the secondary TH2 response (Padigel *et al.*, 2007).

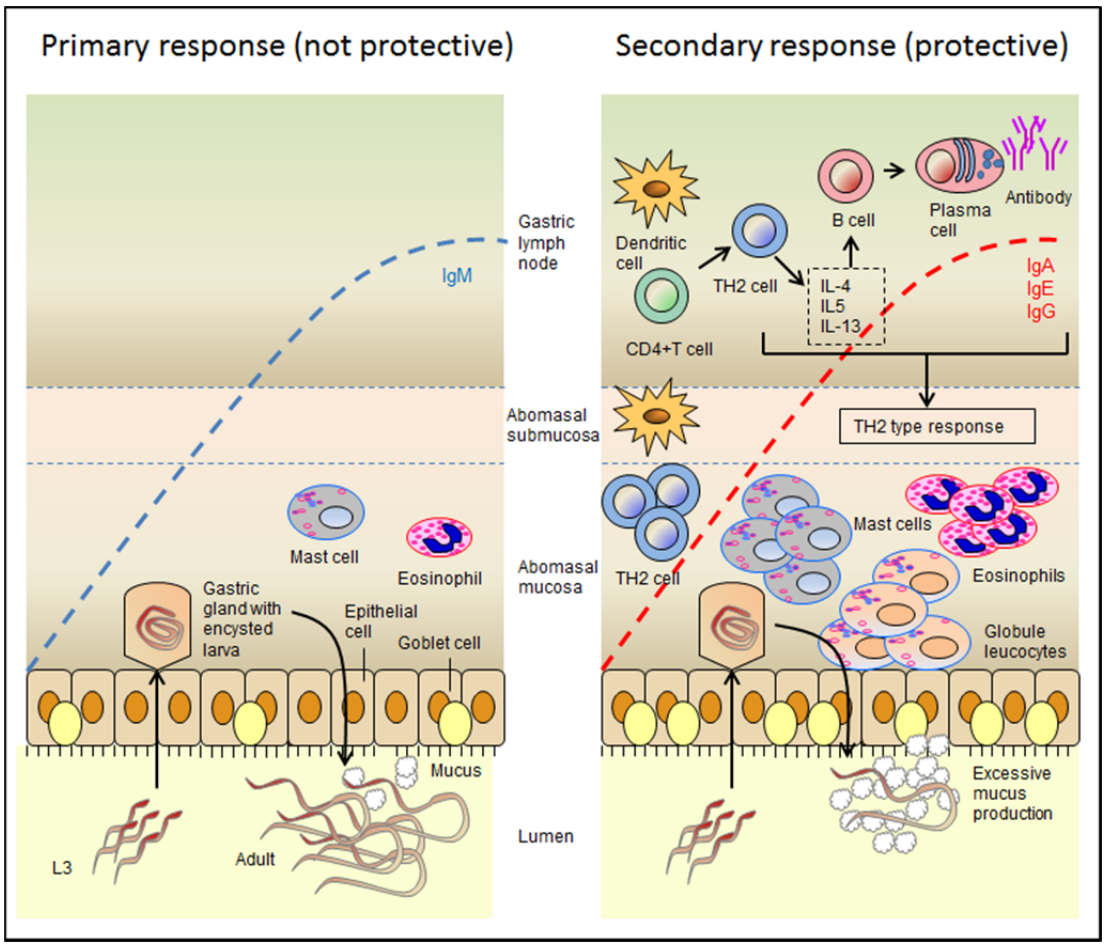


Figure 1.3 Immune response to *Teladorsagia circumcincta* infection. Infective larvae (L3) are ingested by the host and then travel to the mucosal epithelium. L3 becomes L4 when they reach the gastric glands where they moult again to become L5 (immature adult) and re-enter the lumen of the abomasum to become egg-laying adults. Primary infection characterized by high IgM is typically associated with worm establishment while secondary infection with repeated challenge leads to worm clearance/reduction over time. Parasite antigens are processed and presented by dendritic cell to CD4+T cells via MHC Class II in abomasal lymph node. A TH2 type of response is initiated with effector functions mediated by the production of cytokines primarily IL-4, IL-5 and IL-13. Antibody class switching to IgA and IgE is also facilitated by TH2 cells. TH2 effector functions are carried out by eosinophils, mast cells, globule leucocytes, and goblet cells as well as worm-specific antibodies mediated by cytokines.

1.5.1.2 Effector mechanisms of protection

The gut has physiochemical properties that can avert the successful lodgement of larva and adult nematodes. The GI mucus acts as a barrier through its surfactant activity (Belley *et al.*, 1999; Lichtenberger, 1995). Evidence of worm removal with changes in smooth muscle contractility has been demonstrated in mice with *Heligmosomoides polygyrus* (Zhao *et al.*, 2003) and with E/S of *T. circumcincta* (Scott and McKellar, 1998) in sheep *in vitro.* These physiological mechanisms have been linked with IL-4 expression (Anthony *et al.*, 2007). However, none of them have been demonstrated *in vivo* in GI nematodes of sheep, hence it is uncertain if any of this mechanism operates in the expulsion of *T. circumcincta*.

Mast cells are key effectors in TH2 and IgE-mediated protective immune response. Failure of larval establishment or expulsion of adult worm is thought to be hypersensitive reaction mediated by mast cell proliferation (Greer *et al.*, 2008a; Miller, 1996; Stear *et al.*, 1995) and consequent degranulation of mast cells is triggered by IgE, histamine, and cytokines (Kawakami and Galli, 2002; Pochanke *et al.*, 2007). *T. circumcincta* larvae failed to establish with influx of mast cells to the abomasum and draining lymph during the infection (Greer *et al.*, 2008; Stear *et al.*, 1995). In addition, mucosal mast cells (MMC) were elevated in the abomasal mucosa of sheep that are immune to *H. contortus* (Huntley *et al.*, 1992). Significant increase in mast cell proteinase was also seen in gastric lymph of sheep that are immune against *T. circumcincta* (Huntley *et al.*, 1998; Stear *et al.*, 1995).

Accumulation of globule leukocytes, which are degranulated mucosal mast cells (Huntley *et al.*, 1992), have also been linked to rejection of *T. circumcincta* (Stear *et*

al., 1995) and *H. contortus* (Gamble and Zajac, 1992). Taken together, mast cells are essential in mounting protective immunity to GI nematode infections. However, mast cell-independent worm expulsion has been documented such as in *O. ostertagi* infection in cattle (Claerebout *et al.*, 2005) and *T. circumcincta* in sheep (Lacroux *et al.*, 2006).

Eosinophils have been shown to have a significant role in protection to GI nematode infection. Unsuccessful larval establishment was attributed to eosinophilia in *Strongyloides ratti* of mice (Galioto *et al.*, 2006; Watanabe *et al.*, 2000) and *H. contortus* of sheep (Balic *et al.*, 2006; Robinson *et al.*, 2010a). Eosinophilic granules were shown to be capable of killing the larvae (Hamann *et al.*, 1987; McLaren *et al.*, 1981) by adhering to the cuticle (Rainbird *et al.*, 1998; Terefe *et al.*, 2009) which is initiated by recognition of the antibody-bound antigen by the Fc receptors (Martin *et al.*, 2001). Eosinophils seem to act only on incoming larvae as they were found to be closely associated with larvae on tissues 1-2 days after infection but not so after 22 weeks (Balic *et al.*, 2006) and has no effect on adult worm numbers (Henderson and Stear, 2006). However, their role in protective immunity is unclear as other studies show insignificant difference in circulating or tissue eosinophil population of sheep with low or high *T. circumcincta* burdens (Huntley *et al.*, 1995; Schallig and Van Leeuwen, 1997).

Neutrophils, basophils, and alternatively-activated macrophages have also been implicated in protective immune response to helminth infections. Neutrophils may arbitrate nematode killing in gut nematode (Padigel *et al.*, 2007) of mice and promote high level expression of TH2-associated cytokines (Anthony *et al.*, 2007; Morimoto

et al., 2004). Preliminary reports in mouse models have shown that basophils may be required to generate a TH2 response (Perrigoue *et al.*, 2009; Yoshimoto *et al.*, 2009). Current evidence also propose the participation of alternatively activated macrophages (AAMs) (Anderson and Mosser, 2002; Anthony *et al.*, 2007; Wynn, 2008) by immune-regulation (Taylor *et al.*, 2006), tissue repair (Martin and Leibovich, 2005), and limiting parasite invasion (Anthony *et al.*, 2006).

It can be generalized based on the previous discussion that cellular response is crucial to the instigation, continuance, and conclusion of the immune response to GI nematode infections. However, the resolution of infection requires the mobilization of the cells of the adaptive immune system. It can also be concluded that multiple cell types may be involved in enhancing the protective TH2 type of response.

1.5.2 Acquired immune response in helminth infections

1.5.2.1 Antibody response

The distribution of Ig isotypes in ruminants is different to many other species. IgA is most abundant in mucosal secretions the most common isotype in both plasma and mucosal secretions is IgG1 (Conley and Delacroix, 1987; Gill *et al.*, 1992; Simecka, 1998).

Several studies are in agreement that IgA, IgE and IgG may have a possible relationship with resistance to GI nematodes of sheep (Gill *et al.*, 1994; Pernthaner *et al.*, 2006; Schallig *et al.*, 1994; Stear *et al.*, 1995; Strain *et al.*, 2002). However, the majority of these studies looked at serum antibody levels that may not represent the local tissue immune response which is directly involved in the effector mechanism of protective immunity. Furthermore, investigations were limited to establishment of

associations between parameters of resistance and antibody levels, and no reference to the direct IgA activity on the parasite.

Subsequent studies have studied the local immune response by monitoring antibody levels in the efferent lymph by abomasal lymphatic cannulation (Pernthaner *et al.*, 2006; Smith *et al.*, 1983). Measurements of serum and mucus antibody levels for IgG, IgE and IgA were also done following vaccination of lambs with fractionated E/S products of *H. contortus* (Bakker *et al.*, 2004). These studies have shown elevated levels of IgA in vaccinated/immune sheep confirming the important role of IgA in protective immunity to GI nematodes in sheep even in the local site of the immune response. Furthermore, there was a strong relationship between peripheral eosinophils with serum and gastric mucus IgA activity (Martinez-Valladares *et al.*, 2005).

How IgA antibody works in mounting protective immunity against GI nematodes is not clear. There are indications that IgA antibody can control worm development and egg production (Barger *et al.*, 1985; Gill *et al.*, 1994; Halliday *et al.*, 2007). IgA antibody has been associated with the control of worm fecundity (Lacroux *et al.*, 2006; Strain *et al.*, 2002; Strain and Stear, 2001) and worm length of *H. contortus* and *T. circumcincta* and is also implicated in rejection of incoming L3 and delay of larval development (Greer *et al.*, 2008a; Jackson *et al.*, 2004; Jackson *et al.*, 1988). Likewise, there was significant linkage between IgA antibody levels, adult worm numbers, worm length and FEC in resistant and susceptible sheep (Beraldi *et al.*, 2008).

The associations of IgA antibody with protection are thought to be mediated by hypersensitive reactions (Alizadeh *et al.*, 1986; Greer *et al.*, 2008; Jackson *et al.*, 2009) or formation of antibody/immune cell complexes that prevents larval establishment and worm development. An *in vitro* study suggested that larval establishment could be inhibited by eosinophil degranulation via secretory IgA (Abu-Ghazaleh *et al.*, 1989). The experiment demonstrated eosinophil degranulation as indicated by signals from eosinophil-derived neurotoxin (EDN) co-incubated with Ig isotypes. It seems physically unlikely for secretory IgA at the mucosal surface, and therefore outside the body, to bind to eosinophils in tissues. However, human eosinophils have receptors for the secretory component of IgA and binding of this component could trigger eosinophilic degranulation (Gounni *et al.*, 1994). Other mechanisms of protection have been identified including neutralization and inactivation of metabolic enzymes by IgA antibody (Gill *et al.*, 1993a) and the feed-suppressing activity that would result to reduced adult worm length in *T. circumcincta* with IgA (Beraldi *et al.*, 2008; Craig *et al.*, 2007; Smith *et al.*, 1985; Stear *et al.*, 2004).

IgG levels have also been shown to be associated with resistance to nematode infections in a number of studies. Egg production of *H. polygyrus* was reduced in the presence of polyclonal IgG antibodies (McCoy *et al.*, 2008). High IgG levels were associated with reduced worm infection in immunized (Harrison *et al.*, 2008) and genetically resistant sheep (Gill, 1991; Pernthaner *et al.*, 2006). The protective activity of IgG was attributed to neutralizing metabolic enzymes (Smith *et al.*, 1985) and suppressing the ability of the worms to feed (Bottjer *et al.*, 1985).

The localization of mast cells and eosinophils in GI nematode primary infection is suggestive of an immediate hypersensitive reaction (Jackson *et al.*, 2009). Up-regulated IgE activity was observed in sheep protected against *H. contortus* (Kooyman *et al.*, 2000; Shakya *et al.*, 2009; Shakya *et al.*, 2011), *T. circumcincta* (Huntley *et al.*, 1998) and *T. colubriformis* (Shaw *et al.*, 1998) after secondary exposure. IgE up-regulation induced by the larval stage (Huntley *et al.*, 1998) was associated with mast cell activity (Alizadeh *et al.*, 1986) and eosinophil-mediated worm killing (Simecka, 1998).

Indeed, there is compelling evidence that IgA mediates protective immunity to GI nematodes in sheep. Notwithstanding the dearth of information on the direct effect of IgA on the worms, it is still convincing based on current literature that IgA promotes control of worm development. The recent finding on trans-colostral transfer of immunity against *H. contortus* in goats is interesting (Guedes *et al.*, 2010). A negative FEC for up to four months was presented with low IgG and high IgA levels in the serum. This opens another venue for exploring more on the potential of IgA antibody in conferring protection to GI nematodes.

1.5.2.2 T cell response in helminth infections

Cluster of differentiation (CD)4+ T cells are central to the role of protective immunity against nematode parasites (Finkelman *et al.*, 1997). *In vivo* and *in vitro* studies confirm that expulsion of GI nematodes is dependent on induction of T cells and challenge infection in athymic rodents resulted in the delay or non-expulsion of worms (Onah and Nawa, 2000). CD4+ T cell depletion also abated nematode resistance of selected sheep lines (Pena *et al.*, 2006). That CD4+ T cells are

important in the control of immunity to GI nematode infection has been well studied in sheep (Gill, 1994; Gill *et al.*, 1993a; Gill *et al.*, 1993b; Miller and Horohov, 2006; Pena *et al.*, 2006). However, the underlying mechanisms that control infection, leading to susceptibility or resistance, have not been defined.

The expanding range of CD4+ T cell subsets and their tendency towards plasticity (Panzer *et al.*, 2012) emphasizes the dynamics of helminth immunity for both worm and host species and strains. Table 1.2 shows the characteristic features of the CD4+ T cell types associated with protective immunity to GI nematodes.

Table 1.1 Main features of the different CD4+ T helper cell subsets

	TH1	**TH2**	**TH17**	**Treg**
Primary cytokines[8]	IFNγ, IL-12	IL-4	IL-6, TGFβ	TGFβ
Secondary cytokines[9]	IL-2, IFNγ	IL-4, IL-13	IL-17, IL-21, IL-23	IL-10
Autocrine cytokines[10]	IFNγ	IL-4	IL-21	TGFβ
STAT regulators	STAT1, STAT4	STAT5, STAT6	STAT3	STAT5
Lineage specific transcription factors	T-bet	GATA3	RORγt	FOXP3

STAT – signal transducer and activator of transcription; IL – interleukin; TGFβ- transforming growth factor beta; IFNγ – interferon gamma; (FOXP3) – Forkhead box P3; RORγt – retinoic acid orphan nuclear receptor gamma t.

[8] Primary cytokines – direct the differentiation of naïve Tcell to T helper subset
[9] Secondary cytokines – promote proliferation of TH cell subset
[10] Autocrine cytokines – produced selectively by TH cell subset

For GI nematode infection, it is unclear whether the generation of a TH1 response is associated with susceptibility and that resistance/susceptibility is simply a matter of TH1/ TH2 dichotomy (Miller and Horohov, 2006; Maizels *et al.*, 2009). In mice, a highly polarized TH2 response controls *H. polygyrus* (Anthony *et al*., 2007). Host strain also affects the type of immune response. *Trichuris muris* is expelled from Balb/c mice by a polarized TH2 response but the AKR strain become chronically infected in the presence of a TH1 response (Maizels and Yazdanbakhsh, 2003). In contrast, the immunology of *T. circumcincta* control in sheep seems to be distinct from these murine models in that IFNγ expression was unaffected by infection of either 'immune' or 'naïve' lambs (Craig *et al.*, 2007). The CD4+/CD25+/Foxp3+ regulatory T cell subset (Treg) has been shown to be critical for the clinical outcome of helminth infection. Resistance to helminths in mice seems to be determined by a balanced TH1/ TH2/Treg response; unbalanced modified TH2 (high TH2/Treg) and uncontrolled TH1 (high TH1) results in persistent infection and clinical disease (Maizels and Yazdanbakhsh, 2003; Belkaid and Tarbell, 2009).

More recently it has been found that the TH17 CD4+ T cell subset also plays an important role in human and mouse inflammatory bowel diseases as well as host responses to parasites (Weaver *et al.*, 2007; Fouser *et al.*, 2008; Bogaert *et al.*, 2010). Indeed, there seems to be a reciprocal development of Treg and TH17 cells in autoimmune or bacteria-associated inflammatory diseases (Bettelli *et al.*, 2006; Korn *et al.*, 2009; Xu *et al.*, 2011).

The cytokine environment regulates the type of immune response; in mice, resistance to nematodes develops through the regulated differentiation and expansion of

effector cells like eosinophils, mast cells, and antibody-secreting cells (Patel *et al.*, 2009). These mechanisms are thought to be mediated by the cytokine environment as determined by the type of T helper cells that predominate in the immune response.

In this study, I focused on cytokines and markers that are identified with the four major CD4+ TH cell subsets which included: IL-2, IL-4, IL-6, IL-7R, IL-10, IL-12, IL-17A, IL-21, IL-23A, EBI3, FOXP3, IFNγ, and TGFβ. This set was selected on the basis that the differential expression of resistance and susceptibility in study lambs could be defined with the predominance of these transcripts corresponding with the type of immune response. Cytokines may be produced by many different cells and are also known for their redundant functions. Nevertheless, most of them have unique characteristics and may work synergistically or antagonistically with others. Features of these cytokines are described in Table 1.3.

Previous studies that described the immune response to common GI nematode infection in sheep involved acute challenge infection or infect-treat-challenge experiments. In both cases, immune response was monitored at specific time points on cytokines and markers almost exclusive to TH1 and TH2 type of response. In this study we simulated a natural, chronic infection with *T. circumcincta* by trickle infecting lambs for a period of three months with no anthelmintic treatments. It is assumed that maturation of the immune response has developed after this period when the parameters of the immune response were measured.

1.5.3 TH cell types in nematode infections

The existing dogma on the type of immune response to helminth infection is the typical TH1 and TH2 response which corresponds to susceptibility and resistance

respectively (Mosmann and Sad, 1996). In most murine models and human studies, GI nematodes generally trigger a TH2 type protective immune response with the production of signature cytokines IL-4, IL-5 and IL-13. Experiments on mouse intestinal worm parasites *H. polygyrus a*nd *Nippostrongylus brasiliensis* have linked high levels of interleukin 4 (IL4), IL10 and IL13 with resistance and high IL2 and interferon γ (IFNγ) with susceptibility (Maizels and Yazdanbakhsh, 2003; Anthony et al., 2007). Other cytokines associated with the TH2 immune response include IL-21 (Frohlich *et al.*, 2007) and IL-25 (Fallon *et al.*, 2006).

Although discrimination between the two types of immune response based on cytokine profile is not very clear in ruminants, similar studies in sheep using acute challenge with *T. circumcincta* or *H. contortus*, of infected/reinfected ('immune') animals or selected resistant and susceptible lines has confirmed the murine data identifying the TH2 polarized immune response (Miller and Horohov, 2006; Craig *et al*., 2007; Ingham *et al.*, 2008; Meeusen *et al*, 2005; Lacroux *et al*., 2006; Terefe *et al*., 2007; Craig *et al.*, 2007; Shakya *et al*, 2009; Pernthaner *et al*, 2006; Robinsons *et al*., 2010).

Table 1.2 Basic features of cytokines and markers used in defining immune response to *T. circumcincta* (Delves and Roitt, 2011; Murphy *et al.*, 2008)

Cytokine	Producer cell	Action
IL-2	TH1 cells	T and B cell proliferation
IL-4	TH2 cells, natural killer cells, mast cells	B-cell activation, IgE switch, TH2 differentiation
IL-6	TH2 cells, macrophages, endothelial cells, dendritic cells (DC)	T- and B-cell growth and differentiation, TH17 induction, pro-inflammatory
IL-7R	Bone marrow and thymus	Growth of pre-B and pre-T cells
IL-10	Monocytes, macrophage, T and B cells, DC	Potent suppressor of macrophage and TH1 cell functions
IL-12	Macrophage, DC, T and B cells	Activates NK cells, differentiation to TH1
IL-17A	TH17 cells	TH17 proliferation; stimulates production of proinflammatory cytokines
IL-21	TH2 cells	Induces proliferation of B, T and NK cells
IL-23	DC	Expansion and maintenance of TH17 cells, promotes induction of pro-inflammatory cytokines
IL-25	TH2 cells, macrophages, mast cells	Promotes TH2 cytokine production
EBI3	Treg cells	Suppresses inflammatory response
IFNγ	TH1, NK cells	Macrophage activation, Ig class switching, suppresses TH2
FOXP3	Treg nuclear transcriptor	Suppresses T cell proliferation
TGFβ	Chondrocytes, monocytes, T-cells	Inhibits cell growth, anti-inflammatory, induces switch to IgA production

Protective immunity to GI nematode infection is correlated with mucosal mastocytosis, tissue or blood eosinophilia, and parasite-specific IgA, IgE, and IgG (Amarante *et al.*, 2005; Balic *et al.*, 2006; Bricarello *et al.*, 2002; Gill *et al.*, 2000). This is accompanied by the selective expansion of TH2 type CD4+ T helper cells with corresponding down-regulation of TH1 response (Balic *et al.*, 2000b; Behnke *et al.*, 2003; Craig *et al.*, 2007; Gill *et al.*, 2000; Terefe *et al.*, 2007a; Urban *et al.*, 1991; Watanabe *et al.*, 2003). The major characteristics of these cytokines are briefly accounted below.

1.5.3.1 TH1-associated cytokines

The direct role of IFNγ in GI nematode infections has not been established but since its function is to down-regulate IL-4 expression, TH2 proliferation is inhibited while the TH1 response predominates (Pulendran, 2004). Some studies have shown constant or increased expression of IFNγ despite a predominant TH2 response (Meeusen *et al.*, 2005; Pernthaner *et al.*, 2006). It is also interesting to note that IFNγ can work synergistically with IL-4 in the transcytosis of sIgA (Amin *et al.*, 2007). These observations may be attributed to polymorphisms in the IFNγ gene which may alter the immune response (Coltman *et al.*, 2001a).

IL-12 initiates the development of TH1 by driving naïve CD4+ T cells to TH1 cells and amplifies the expression of IFNγ. IL-12 promotes a chronic intestinal nematode infection (Allison *et al.*, 1997; Khan *et al.*, 2001).

IL-2 plays a central role in TH2 differentiation by stabilizing the accessibility of IL-4 to STAT5 (Cote-Sierra *et al.*, 2004). It is also an important activator of Treg

suppressive activity and increased IL-10 production (Brandenburg *et al.*, 2008; De La Rosa *et al.*, 2004).

Epstein-Barr virus-induced gene 3 (EBI3) is a widely expressed interleukin-12p40-related protein that is essential in the regulation of effector T cells and inflammatory responses. It is derived from the heterodimeric cytokine IL-27 that consists of EBI3, IL27 p28, a newly discovered IL-12p35-related polypeptide (Pflanz *et al.*, 2002) EBI3 has been reported to be elevated in IBD secondary to influx or local proliferation of inflammatory cells (Gehlert *et al.*, 2004). Initial studies suggested that it had an important role in promoting Th1 responses but subsequent studies have revealed that EBI3 receptor signalling influences a variety of immune cell types and can inhibit both Th1 and Th2 responses (Dokmeci *et al.*, 2011; Nieuwenhuis *et al.*, 2002; Zahn *et al.*, 2005). It was also found to have inhibitory effects on Th17 T cells (Yang *et al.*, 2008a) and may contribute to the activity of Treg cells as a downstream target of FOXP3 (Collison *et al.*, 2007).

1.5.3.2 TH2-associated cytokines

IL-4 is critical to the signalling pathway that differentiates naïve CD4+ T cells into TH2 phenotype through STAT6 which involves up-regulation of GATA3 (Fallon *et al.*, 2002; Zheng and Flavell, 1997). IL-4 and IL-13 have been shown to mediate worm expulsion through increased mucosal permeability, and enhanced mucus production and muscle contractility (Madden *et al.*, 2002; Meeusen *et al.*, 2005; Anthony, 2007). These cytokines also promote B cell responses, including isotype switching from IgM to IgA and IgE (Maizels and Yazdanbakhsh, 2003).

IL-5 and IL-13 are important in eliminating GI nematode parasites as IL-5 stimulates and activate eosinophils while IL-13 promotes antibody class switching to IgE. Up-regulation of these two cytokines after *T. colubriformis* challenge (Lacroux *et al.*, 2006; Meeusen *et al.*, 2005) coincided with increase IgE production (Kooyman *et al.*, 2000) and greater number of eosinophils (Henderson and Stear, 2006). In addition, it has been shown both *in vitro* and *in vivo* that IL-5 influences the differentiation of IgA-producing plasma cells (Ramsay *et al.*, 1994b). However, other mechanisms can compensate for IgA synthesis in the absence of IL-5 (Simecka, 1998).

IL-25 also known as IL-17E is produced by activated TH2 cells and mast cells. Owyang *et al.*(2006) have identified two major roles of IL-25 in immune regulation: promoting TH2 response by driving the expression of IL-4, IL-5 and IL-13 (Fallon *et al.*, 2006; Fort *et al.*, 2001) and limiting the production of pro-inflammatory cytokines and chronic pathologic inflammation at mucosal sites. Indeed, IL-25 (-/-) developed severe intestinal inflammation with elevated levels of IFNγ and IL-17 in *T. muris* infection (Owyang *et al.*, 2006).

IL-10 was initially thought of as a TH2-type cytokine (Gause *et al.*, 2003; Iwasaki and Kelsall, 1999) but recent findings show that it is not exclusive to this type of response; TH1 and Treg cells (Anderson *et al.*, 2007; Beiting *et al.*, 2007; Elliott *et al.*, 2004) as can macrophage and DCs produce IL-10.

1.5.3.3 Treg cells

TH2 responses to GI nematodes are generally protective but uncontrolled effector mechanisms may result to pathology (D'Elia *et al.*, 2009; Maizels and Yazdanbakhsh, 2003). CD4+ Treg cells protect against this consequence (Tang and

Bluestone, 2008) by their ability to actively suppress the immune response (Belkaid and Rouse, 2005; Sakaguchi, 2000). Treg cells strike a balance that reduces immune-mediated pathology to the host while still allowing sufficient immune response against the parasite (Belkaid and Tarbell, 2009).

CD4+ Treg cells are usually identified as expressing CD25 (the α chain of the IL-2 receptor) and the nuclear transcriptor FOXP3 (Fontenot *et al.*, 2005; Hori *et al.*, 2003). FOXP3 is in control of Treg lineage (Zheng and Rudensky, 2007) particularly with chronic helminth infection. In chronic *H. polygyrus* infection, a TH2-dominated cytokine profile was observed from 7-14 days of infection which shifted to a regulatory T cell response by day 28. The regulatory profile was marked by expansion of FOXP3+ cells and elevated IL-10 and high frequency of TGFβ-expressing CD4+ T cells (Finney *et al.*, 2007) which are mediators of Treg cell differentiation.

Much of the work on Treg cells had involved cancer, allergy and autoimmune diseases in mice and humans. For helminth infection, Treg cell activity has been demonstrated in human infections with filarial worms *Onchocerca volvulus* (Korten *et al.*, 2008) and *Litosomoides sigmondontis* (Taylor *et al.*, 2005) and in the mouse strongyle *H. polygyrus* (Finney *et al.*, 2007). Treg cell activity was also observed in paratuberculosis characterized by IL-10 up-regulation and consequent suppression of TH1 response in cattle (De Almeida *et al.*, 2008) and sheep (Smeed *et al.*, 2007). Treg cells were also implicated with increased IL-10 and TGFβ expression in the abomasal lymph node of *T. circumcincta* infection (Craig *et al.*, 2007) and in the lymph of sheep resistant to *H. contortus* and *T. colubriformis* (Ingham *et al.*, 2008)

1.5.3.4 TH17

CD4+ TH17 cells have emerged as a distinct effector cell subset that produces IL-17A and not IFNγ and IL-4, which define TH1 and TH2 lineage respectively. A TH17 type of response is associated with inflammatory (Boniface *et al.*, 2008) and autoimmune diseases (Weaver *et al.*, 2006) like inflammatory bowel disease (IBD) and MS (Wilson *et al.*, 2007). In mice, TH17 cells express the transcription factor retinoic acid orphan receptor (ROR) γt (Yang *et al.*, 2008d) and STAT3 as the signal regulator. IL-6 is critical to TH17 development as it drives the TGFβ-induced naive T cells to differentiate to TH17 instead of Treg lineage (Mangan *et al.*, 2006; Veldhoen *et al.*, 2006). The other pathway involves TGFβ and IL-21 combination to signal differentiation of TH17 in the absence of IL-6 in mice (Korn *et al.*, 2007) and in humans (Yang *et al.*, 2008b). IL-6 appears to be indispensable in the TH17 type of response as it not only activates STAT3 which is critical to TH17 differentiation, but also up-regulates IL-23R and works synergistically with IL-23 (Morishima *et al.*, 2009; Yang *et al.*, 2007).

TH17 were initially thought to be related to TH1 but are now considered a fixed CD4+ phenotype independent of the TH1 cell type (Annunziato and Romagnani, 2011). IL-23 shares the common p40 subunit with IL-12; both are capable of inducing IFNγ production. However, IL-23 is distinguished from IL-12 by its ability to amplify and stabilize the expansion of TH17 cells which produce IL-17A (Iwakura and Ishigame, 2006). The relationship between TH1 and TH2 is further supported by TH17 committed cells being induced to differentiate into TH1; TH17 cells were re-polarized to TH1 differentiation in the presence of IL-12 (Annunziato *et al.*, 2007).

IL-23 is a novel pro-inflammatory cytokine that has been linked to several autoimmune diseases and inflammatory pathology (Boniface *et al.*, 2008; Iwakura and Ishigame, 2006). IL-23 activates STAT3 differentiation (Yang *et al.*, 2007) and stimulates IL-17 production and expression (Iwakura and Ishigame, 2006). Variants of IL23R gene have been associated with susceptibility to IBD (Duerr *et al.*, 2006; Lacher *et al.*, 2010). On account of this, IL-23 is targeted as a potential drug therapy for IBD, as it was shown to be enormously increased in affected patients (Tang *et al.*, 2012).

IL-21 is essential for amplification of TH17 cells (Korn *et al.*, 2007; Nurieva *et al.*, 2007). TH17 cells selectively produce IL-21 which activates STAT3, and induces expression of RORγt, IL-17A and IL-17F thus; promoting further lineage commitment of TH17 cells (Wei *et al.*, 2007). IL-21 serves as a key modulator of TGFβ signalling, which leads to TH17 or Treg differentiation (Fantini *et al.*, 2007). IL-21 has been known to regulate B cells particularly its transition to antibody-producing plasma cells (Ettinger *et al.*, 2005; Ozaki *et al.*, 2002).

IL-17 which is secreted chiefly by CD4+ T cells stimulates the production of IL-1β, IL-6, tumour necrosis factor-α (TNFα) and chemokines responsible for inflammation (Iwakura and Ishigame, 2006). TH17 and IL-17A are also associated with immune-pathologies in infectious diseases like tuberculosis, toxoplasmosis and schistosomiasis (Gaddi and Yap, 2007; Umemura *et al.*, 2007; Wen *et al.*, 2011).

Whilst TGFβ has been identified to be involved in the induction of TH17 differentiation (McGeachy *et al.*, 2007; Veldhoen *et al.*, 2006; Yang *et al.*, 2008b), other studies have considered TGFβ to be not critical to human TH17 cell

development (Ghoreschi *et al.*, 2010). Deletion of TGFβ did not affect the proportion of gut TH17 cells (Gutcher *et al.*, 2011) and cytokines IL-23 and IL-6 were able to induce IL-17A production in the absence of TGFβ (Qin *et al.*, 2009). As TGFβ is ubiquitous especially in mucosal tissues, it is difficult to establish its association to TH cell differentiation. Whether TGFβ promotes TH17 differentiation or it suppresses the expression of other cytokines or transcription factors associated with other TH cell lineage is not clear. The anti-inflammatory Treg cells are thought to interact with the pro-inflammatory TH17 cells as both have preferential localization at mucosal surface and require TGFβ to develop from antigen-naive T cells (Weaver and Hatton, 2009). TGFβ-dependent expression of FOXP3 can be blocked by IL-21 (Fantini *et al.*, 2007) and IL-6 resulting in TH17 instead of Treg cell differentiation FOXP3 which is the transcription factor of Tregs is inhibited by IL-6 (Bettelli *et al.*, 2006; Fallon *et al.*, 2006; Mangan *et al.*, 2006).

IL-7R plays a critical role in T lymphocyte development. It is genetically associated with susceptibility to multiple sclerosis (MS) while IL-7 has been shown to directly expand effector TH17 cells in experimental autoimmune encephalitis (EAE) as well as MS (Liu *et al.*, 2010). Polymorphisms in IL-7R have been identified as risk factor for many autoimmune diseases like multiple sclerosis (MS) and inflammatory bowel disease (IBD) suggestive of the IL-7 pathway as a target for drug therapy (Mazzucchelli *et al.*, 2012).

1.6 Aims and importance of the study

This project is a development of a previous study that showed a spectrum of response in naïve Blackface lambs with predicted genetic variability for resistance and

susceptibility to the GI nematode *T. circumcincta* (Beraldi *et al.*, 2008). The experiment focused on the phenotypic analysis of the cohort of lambs. The lambs had been trickle-infected with L3 to simulate natural chronic infectio. This has resulted in animals with a range of susceptibilities that reflected the nature and magnitude of the mature immune response. At one end of the spectrum were lambs with low faecal egg count and adult worm burden, and high IgA antibody and body weight. At the other end of the spectrum were smaller lambs with high FEC and adult worm burden, and low IgA antibody. Lambs with zero to low FEC were defined as resistant while those with high FEC were defined as susceptible. Adult worms were highly aggregated in a few susceptible lambs and absent in resistant lambs, while the early arrested larvae (EAL4) were uniformly distributed across the flock.

I hypothesized that the spectrum of response in lambs has an immunological basis and that the expression of the immune response can be quantitatively correlated with phenotypic parameters of resistance. Since orchestration of the different TH cell affects the consequent immune response, four subsets of CD4+ T cells (TH1, TH2, TH17, Treg) were investigated. This was done by measurement of the transcripts of their characteristic markers and effector cytokines and quantitative expression correlated with individual traits of resistance and susceptibility. The study tested this hypothesis with the following specific objectives:

1. To quantify expression of genes associated with the different CD4+ T cell subsets in order to determine differential activation of Treg and TH17 cells as well as the interaction between TH1 and TH2 subsets in resistant and

susceptible lambs.

2. To develop an optimized real-time RT-qPCR assay for quantifying expression of a set of cytokines and markers in sheep.

3. To correlate differentially-expressed genes in resistant/susceptible lambs with phenotypic traits.

4. To describe the abomasal pathology associated with *T. circumcincta* infection in lambs in known resistant and susceptible lambs.

5. To determine the presence of mutations in genes found to be differentially-expressed in resistant/susceptible lambs.

The study formed part of a big project with the ultimate aim to identify molecular markers for marker-assisted selection of sheep with resistance to *T. circumcincta*. To attain this long-term goal, this study explored the dynamics of T cell immunity during *T. circumcincta* infection for possible identification of candidate gene marker.

Chapter 2 Materials & Methods

2.1 Animal experiment and tissue sample collection

2.1.1 Source of tissue samples

Tissue samples used in the study were sourced from a previous experiment (Beraldi *et al*., 2008) on 57 Blackface lambs bred at the Roslin Institute (Edinburgh, UK).. The parents of the study lambs belonged to a Blackface sheep population used previously for quantitative genetic and quantitative trait loci (QTL) analyses of FECs (Davies *et al.*, 2006) and estimated breeding values for FECs of the parents were available. The dams available for mating were grouped into those above and below the mean of the sample. The mean breeding value for log-transformed FECs for the top-half dams was 0.193 (SD = 0.160, n = 23) while for the bottom-half the mean was 0.573 (SD = 0.138, n = 19). The three sires had breeding values of 0.468, 0.228 and 0.063. In order to spread genetic variation in parasite resistance across the progeny, each sire was mated with an equal number of dams from the top-half and bottom-half of the breeding value distribution. The lambs had been kept as parasite-free as possible by preventing them from grazing on pasture until the start of experimental infection. Pregnant ewes were had been brought indoors and treated with three different parasiticides, moxidectin (Cydectin, 1 ml/5 kg body weight), levamisole (Levacide, 0.5 ml/2 kg), and fenbendazole (Panacur, 2.5–3 ml/sheep) After weaning at about three-month of age lambs had been infected with approximately 2,300 *Teladorsagia circumcincta* infective larvae (infected group) or sham-dosed (uninfected control) three times weekly until sacrifice (12–14 weeks). The phenotypic data (FEC, AWC, IgA antibody levels, and body weight) used in the current study were generated from the experiment (Beraldi *et al*., 2008) described. Individual FEC was determined by modified McMaster technique (MAFF, 1968) two

weeks before infection and on alternate weeks thereafter until sacrifice. Adult worm counts were calculated from aliquots of the abomasal contents collected at post-mortem. Body weight was recorded at the start of the experiment (Day 0–2) which continued every two weeks until sacrifice (Figure 2.1). Antibody capture ELISA in the serum has been performed to determine anti-*T. circumcincta* IgA measurements.

Faeces for FEC were collected from each animal two weeks before infection and on alternate weeks thereafter until sacrifice while adult worm numbers were determined post-mortem. Blood values and body weight were recorded at the start of the experiment (Day 0–2) which continued every two weeks until sacrifice (Figure 2.1).

2.1.2 Tissue collection and preservation

The lambs were sacrificed after approximately 3.5 months post-infection. Tissue blocks of abomasum and gastric lymph nodes measuring 5 mm thick were collected from each animal. The tissue samples were either preserved in RNAlater® (Ambion, UK) prior to storage in -80°C for RNA extraction, or fixed in Zinc Salt fixative for histopathology (Gonzalez *et al.*, 2001).

2.1.3 Histopathology

Fixed tissue samples were sent to the Veterinary Pathology Unit, R(D)SVS, University of Edinburgh for processing into 5 µm thick sections stained with routine haematoxylin and eosin or toluidine blue for mast cell quantification.

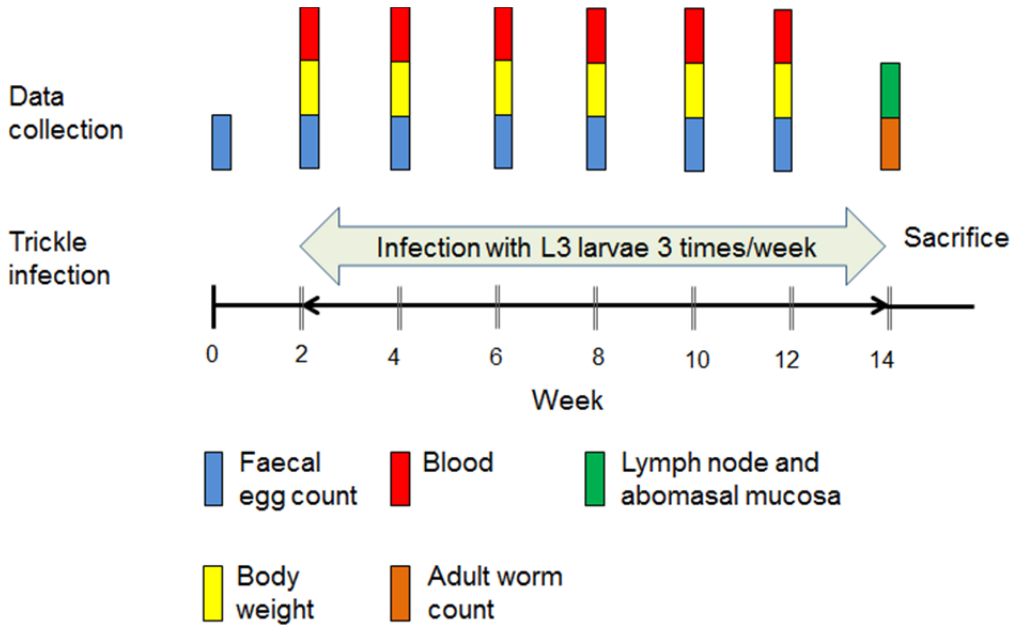


Figure 2.1 Experimental design and sampling schedule for phenotype analysis in lambs. Resistant and susceptible lambs were infected with 2,300 L3 infective larvae three times a week until three days before sacrifice; non-infected controls were sham-dosed. Phenotype data were collected and evaluated every two weeks (Beraldi *et al.*, 2008). Adult worm count was determined post-mortem. Abomasal lymph node and abomasal mucosa were also collected for RNA extraction.

2.2 RNA Methods

2.2.1 RNA Extraction from abomasum and lymph node

Total RNA was extracted using the protocol in the RiboPure™ kit (Ambion, UK) for abomasal lymph nodes, and RNeasy Mini Kit for abomasal mucosa tissue (Qiagen®). All samples were DNase I digested using Turbo DNA–free (Ambion, UK) as instructed in the kit (Section 2.2.1.2). This was used as the starting material in the study (Table 1, Appendix 1).

2.2.1.1 Quantification and assessment of RNA/DNA purity by UV absorbance

The concentration of RNA/DNA was determined using the Nanodrop® ND-1000 spectrophotometer (Nanodrop Technologies, Inc). A 1.2 µl aliquot of nuclease-free water was selected as the zero absorbance reference. Another 1.2 µl aliquot of sample was loaded onto the measurement pedestal and the absorbance was measured over a continuous spectrum producing a graph. The A_{260}/A_{280} and A_{260}/A_{230} ratios were calculated; an A_{260}/A_{280} ratio in the range of 1.8 and 2.0 is generally acceptable as pure for DNA and RNA respectively. The A_{260}/A_{230} ratio is used as a secondary measure of nucleic acid purity; values outside the 2.0–2.2 may suggest organic chemical contamination such as phenol and thiocyanates. Data on quantification and assessment of extracted RNA from the lymph node and abomasal tissue samples are presented in Table 1, Appendix 1.

2.2.1.2 DNase treatment of RNA

The Turbo DNA-free™ kit (Ambion) was used for DNase re-treatment of RNA samples to remove contaminating genomic DNA. This was done in samples that

tested positive for DNA contamination in RT-minus reactions (Section 2.2.2). A 0.1 µl volume of 10x Turbo DNase buffer and 1 µl Turbo DNAse I (2 U) were added to each tube containing eluted RNA. The tubes were incubated for 30 min at 37°C. A 0.1 µl volume of re–suspended DNase inactivation reagent was added then incubated further for 5 min at room temperature with mixing. The tubes were then centrifuged at 10,000 x g for 1 min to pellet the inactivation reagent. The supernatant was collected into microtubes and stored at -80°C.

2.2.1.3 Evaluation of RNA quality and integrity by Agilent® bioanalyzer

The quality and integrity of the RNA samples were analysed using the Agilent® bioanalyzer with the RNA 6000 Nano LabChip kit (Agilent Technologies). This procedure assesses the RNA quality based on the concentration, ribosomal ratio and assessment of possible DNA contamination. It also gives an RNA Integrity Number (RIN) for eukaryote total RNA as basis on evaluating the degradation of products. The RIN ranges from 1 (lowest integrity) to 10 (highest integrity); the cut-off value for the RIN in this experiment is 6.0 (Fleige and Pfaffl, 2006).

All reagents for chip preparation were allowed to equilibrate to room temperature for 30 min prior to use. The chip was prepared by placing 550 µl of RNA 6000 Nano gel into a spin filter tube then centrifuged at 1,500 x g for 10 min. Gel-dye mix was prepared by mixing 65 µl of filtered RNA 6000 Nano gel matrix with 1 µl RNA 6000 Nano dye concentrate. The gel-dye mixture was centrifuged again at 10,000 x g for 10 min.

A new RNA chip was placed on the Chip priming station. A 9 µl aliquot of Gel-Dye mix was pipetted in the well marked (G). The chip was pressurized before loading 9 µl aliquot of gel-dye mix into the two wells marked (G). A 5 µl aliquot of RNA 6000 Nano marker was added in all 12 sample wells and in well marked (#). An additional 1 µl had to be loaded in unused wells. The RNA samples and the RNA 6000 Nano ladder aliquot were heated to 70°C in water bath for 2 min to denature any secondary structure. A 1 µl aliquot of ladder was added to the well marked (#), then 1 µl RNA was added to each of the sample wells. The chip was vortexed at 2,400 rpm for 1 min on an IKA chip vortex then positioned into the Agilent machine. The required RNA Nano assay was set on the Agilent 2100 Expert software (total RNA Nano) and the assay was performed.

2.2.2 Reverse transcription

Superscript III First Strand Synthesis system for RT-PCR Kit (Invitrogen™) kit was used to synthesize first strand cDNAs from the total RNA samples extracted previously. A 1 µl aliquot of oligonucleotide (dT) primers and 1 µl of dNTP mix (10 mM each) were added to 0.5-1 µg of total RNA in 10 µl final volume. The tubes were heated to denature at 65°C for 5 min and then cooled on ice for 1-5 min. A cDNA synthesis master mix containing 2 µl of 10x RT Buffer, 4 µl 25 mM $MgCl_2$, 2 µl 0.1M DTT, 1 µl 40 U/µl RNaseOut and 1 µl Superscript™ III RT (200 U/µl) was prepared. A 10 µl aliquot of the master mix was added to each RNA/primer mixture. The reaction was incubated at 50°C for 50 min followed by 5 min at 85°C to inactivate the enzyme. Reverse transcription without transcriptase (RT-minus), which was substituted with nuclease-free water was done as control experiments to

detect products amplified from genomic DNA contamination which would interfere with qPCR applications.

2.3 Polymerase chain reaction (PCR)

2.3.1 Design of oligonucleotide primers

Primers used for PCR and quantitative real-time RT-PCR (RT-qPCR) are presented in Table 3.1 and 3.2. RT-qPCR runs for IL-6, IL-10, IL-12B, IFNγ and TGFβ1 were performed using primers designed previously (Smeed et al., 2007; Gossner et al., 2011). The assay for these transcripts was re-optimized and the other transcripts worked out to suit an optimum quantification assay for the samples to be analysed in the current study. Where available, ovine-specific sequences were used (http://www.ncbi.nlm.nih.gov/). Otherwise, sequences from the bovine species were utilized for the amplification of sheep sequences. Sheep amplicons were cloned and sequenced; these sequences were then used to design ovine-specific primers. Primers for the genes of interest were designed either manually or by using Primer 3 Plus (Untergasser *et al.*, 2007). Since primers will be used for downstream RT-qPCR applications stringent criteria were set which include: product size 100-200 bp, primer size 18-24 bp, primer Tm 55-60°C with a maximum difference of 2°C, and GC content of 40-60 %. Other primer features that were considered were the maximum self and 3′-complementarity, and Poly-X (i.e. repeats of any nucleotide in the primer sequence) that was set at 2.0-3.0.

The designed primers were analyzed for secondary structure and dimerization in NetPrimer software (http://www.premierbiosoft.com/netprimer/index.html) with targeted score of 100 or nearest. More emphasis was placed on the similarity in Tm,

GC content, and ΔG between the primers. Primers forming hairpin loops, dimers, cross-dimers, palindromes and repeats were avoided as much as possible.

Upon selection of suitable primers, specificity was checked using BLASTn (Altschul *et al.*, 1990). Highly similar nucleotide sequence was searched to ensure that they were specific for the gene that they were designed for.

2.3.2 PCR protocol

PCR was performed using a PCR Sprint Thermocycler in a final volume of 50 µl. Temperature gradient PCR was performed in PX2 Thermal cycler® (Thermo Electron Corp.). A typical reaction mix was set on ice which consisted of 5 µl 10x *Taq* DNA Polymerase buffer (Promega) with or without $MgCl_2$ (final concentration of $MgCl_2$ varied from 2.0-4.0 mM), 1 µl dNTP mix (to a final concentration of 200 µM each of dATP, dCTP, dGTP, and dTTP), 1 µl of forward and reverse primer at 500 µM, 0.4 µl Taq DNA polymerase (5 U/µl), and 0.5-2.0 µl diluted template DNA synthesized from 0.5-1.0 µg of RNA. The reaction was made to 50 µl total volume with nuclease-free water.

The programme was set to heat the samples to 94-95°C for 2 min for the initial denaturation. The second stage was the thermo-cycling phase, which consisted of a further denaturation of 30-60 sec at 94°C, annealing at an appropriate temperature determined for each gene for 30-60 sec and an extension at 72°C for 2 min. This was usually repeated for 30-35 cycles before the final extension of 5-7 min at 72°C.

2.3.3 Agarose gel electrophoresis and examination in transilluminator

PCR products were visualized by agarose gel electrophoresis. The gels (1.0-1.2%) were prepared by dissolving LE Seakem®, agarose powder (Cambrex Bio Science) into 1 % Bionic buffer (Sigma, Aldrich) and heated until the agarose was dissolved completely. The solution was cooled to ~50°C then 0.04 µl /ml of GelRed™ Nucleic Acid Gel Stain (Biotium) was added before pouring the gel into the tray to allow it to set.

The gel tray was fitted into the electrophoresis chamber and submerged in 1x Bionic™ buffer. Samples were mixed with 6x loading dye (Promega) before loading into wells alongside DNA ladders at 100 bp size. Electrophoresis was carried out at 150V for ~30 min or until DNA separation was achieved. The product size was determined through visualization of bands under UV light using UV-transillumination advanced imaging system (Alpha Innotech Fluorchem® HD2).

2.3.4 PCR product column purification

The MinElute PCR Purification Kit (Qiagen®) was used to clean up the amplified DNA fragment in the PCR product. 5x volume of buffer PBI was added and mixed to each volume of PCR product. The solution was applied onto the silica membrane of the spin column, and then centrifuged at 16,000 x g for 1 min (Biofuge Pico, Heraeus). The flow through was discarded and the columns were washed with 750 µl buffer PE by centrifuging for another minute. The flow through was discarded again and the columns centrifuged for a further 1 min before being transferred into new microtubes. Elution buffer (10 mM Tris-HCl, pH 8.5) was applied onto the

silica membrane, and then centrifuged at 16,000 x g for 1 min to elute the product. The eluted PCR product was quantified as described in Section 2.2.1.1.

2.3.5 PCR product gel extraction

For some genes, more than one DNA product was generated in the PCR reaction, which was revealed by gel electrophoresis (Section 2.3.3). In these cases, products were purified by gel extraction using QIAquick Gel Extraction microcentrifuge protocol (Qiagen®). The target DNA fragment was excised from the agarose gel with a scalpel. The gel slice, which weighed ≤ 400 mg was placed in a microtube, where 3 volumes of buffer QG to 1 volume of gel were added. The gel was incubated at 50°C for 10 min to allow it to dissolve completely. A gel volume of isopropanol was added and mixed with the sample before loading on a column then centrifuged for 1 min at 16,000 x g. The flow through was discarded and the column was placed back in the same tube. The sample was washed with 0.75 ml Buffer PE then centrifuged for 1 min at 16,000 x g. The flow through was discarded and the tube was returned to the same tube and spun again as in previous step to remove any ethanol residue. The column was then transferred to a clean microcentrifuge tube. To elute DNA, 50 µl of Buffer EB was added and allowed to stand for a minute, and then centrifuged for another minute.

2.4 Cloning and sequencing of ovine transcripts

2.4.1 Ligation

Purified PCR products were ligated and transformed using the pGEM®-T Easy vector kit (Promega). The ligation reaction was set up in 0.5 ml microcentrifuge

tubes (Axygen) which consisted of 5 µl of 2x ligation buffer, 1 µl pGEM®-T Easy vector (50 ng), 0.1-0.3 µl of PCR product (at insert:vector ratio of 1:1 and 3:1), 1 µl T4 DNA Ligase, and deionized water to a final volume of 10 µl. The reaction was mixed by pipetting, and then incubated at room temperature for 1 hr. Alternatively, incubation was done overnight at 4°C to increase the yield of transformants.

2.4.2 Transformation in competent cells

The recombinant DNA was allowed to replicate in *E. coli* JM 109 competent cells by mixing with the ligation reactions. A 4 µl aliquot of the ligation reaction was transferred to 15 ml Falcon tubes (Becton Dickinson Labware) on ice. *E. coli* JM 109 High Efficiency Competent Cells (Promega) were removed from -80°C storage and allowed to thaw in an ice bath for about 5 min. A 20 µl aliquot of competent cells were transferred into each tube and placed back on ice for 20 min. Heat shock was applied to the cells by immersing the tubes in water bath for 45 sec at 42°C before cooling back in ice for 2 min. A 980 µl of LB broth (Appendix 2) was added in each tube with the competent cells-ligation reaction mixture. The cells were incubated for 1.5 hrs at 37°C in orbital shaker at 200 rpm. Each transformation culture was plated on LB/ampicillin/IPTG/X-Gal agar plate (Appendix 2) then incubated overnight at 37°C.

2.4.3 Plasmid DNA extraction

Plasmid DNA was isolated using the QIAprep® Spin Miniprep kit (Qiagen®). Colonies were picked from the agar plate then inoculated in 5 ml LB broth with 10 µl ampicillin (Appendix 2). The inoculants were put in a shaker incubator overnight at

37°C. The bacterial cultures were pelletized by spinning at 4,000 rpm (2500 x g) for 5 min then the supernatant was poured off. The pelleted bacterial cells were re-suspended in 250 µl buffer P1 with added RNAse A then transferred to a new tube. The cells were lysed with 250 µl buffer P2 (containing NaOH/SDS), and then neutralized by adding 350 µl buffer N3 (containing guanidine hydrochloride and acetic acid). In between these steps, the tubes were inverted six times to mix. The tubes were then centrifuged at 16,000 x g for 10 min and the supernatants were transferred onto QIAprep spin columns. To bind the plasmid DNA, the tube was centrifuged for 1 min at 16,000 x g, and the flow through was discarded. This was followed by washing with 500 µl buffer PB (containing guanidine hydrochloride and isopropanol) and centrifuged for 1 min at 16,000 x g. The flow through was discarded before adding 750 µl buffer PE to each column. A further 1 min centrifugation was done to remove any residual buffer. To elute the DNA, the column was transferred in a 1.5 ml collection tube, to which 50 µl of buffer EB was applied before setting in the centrifuge at 16,000 x g for 1 min. The eluted DNA was evaluated for purity and concentration by UV absorbance (Section 2.2.1.1).

2.4.4 Digestion of plasmid with restriction enzyme

The restriction enzyme used for each plasmid DNA was based on the detailed restriction map generated by the Sequence Manipulation Suite (SMI) software (http://www.bioinformatics.org/sms2/). Restriction enzyme digestion was set up in total volume reaction of 20 µl. All reagents used were from New England Biolabs. The reaction was composed of 2 µl 10x restriction enzyme buffer, 0.1 µl BSA (if required), 0.5 µg DNA, 0.5 µl restriction enzyme (2-10 U), and nuclease-free water.

The components were mixed by pipetting and then the tubes were pulse-spun before loading in water bath for incubation. The tubes were left in the bath for 1.5 hrs at 37°C. Aliquots of the restriction digest were size-fractionated in routine agarose gel (Section 2.3.3) and visualized under UV trans-illumination.

2.4.5 DNA Sequencing

The BigDye® Terminator v 3.1 Cycle sequencing kit (Applied Biosystems, Warrington, UK) was used to sequence pGEM T-easy plasmids in three independent reactions from each clone in forward and reverse directions. The sequencing reaction was set up at 10 µl total volume mix using the following components: 0.5 µl Big Dye, 1.75 µl 5x sequencing buffer, 3.2 µl sequencing primer (SP6 or T7) , equivalent amount of 200-500 ng for cloned plasmids and 2-5 ng for purified PCR product of DNA template, and nuclease-free water. The reaction was incubated in the PCR machine as follows: 96°C for 10 sec, 50°C for 5 sec, and 60°C for 2 min, for 30 cycles. ABI 3730 capillary sequencing of samples was performed at the GenePool (http://genepool.bio.ed.ac.uk/).

2.4.6 Analysis of sequences

Three independent sequences were obtained for each clone and primers. All sequences were checked with Basic Local Alignment Search Tool (BLAST) (Altschul *et al.*, 1990) to ensure that they contained the proper insert. In case of mismatches, sequences were generated either from other clones in the same culture plate or from new set of clones. Consensus alignment with bovine or ovine sequences, where available, was performed using ClustalW2 (Larkin *et al.*, 2007).

All nucleotide sequences generated and used in the study are presented in Table 2 and 3, Appendix 1.

2.4.7 Linearization and purification of plasmid DNA

NdeI or NcoI enzymes (New England Biolabs) were used to linearize purified plasmid DNA of target genes. The reaction was set up with 2 µl of restriction enzyme buffer, 1-2 µl of restriction enzyme, 500 ng of pDNA and nuclease free water to make up 10 µl total volume. The mixture was incubated overnight at 37°C. A 2 µl aliquot of the plasmid was run on 0.8 % agarose gel to check if linearization was complete.

Linearized plasmids were cleaned-up using MinElute Reaction Cleanup kit (Qiagen®). A 300 µl of buffer ERC was mixed with the pooled reaction then transferred into MinElute columns. This was centrifuged at 16,000 x g for 1 min before adding 750 µl of buffer PE, and then spun again for 1 min. The flow through was discarded then centrifuged again for 1 min. The column was then placed in a new collection tube where 10 µl of buffer EB was added to elute the DNA. Another 1 min centrifugation step was done before collecting the eluted DNA. A 2 µl aliquot of the cleaned-up linearized plasmid was run on 0.8 % agarose gel to confirm that the linearization was complete.

2.5 Optimization of RT-qPCR for sheep tissues

2.5.1 Generation of standard curves from plasmids of DNA clones

All reactions were prepared using CAS-1200 Precision Liquid Handling System and perfomed on the Rotor-Gene™ RG-3000 and The Rotor-Gene™ -Q in combination

with Rotorgene software version 2.02 (Corbett Life Science), and SYBR Green I (Biogene) detection system.

DNA from linearized plasmids (pDNA) from clones of target gene sequences were used to generate standard curves. A total reaction volume of 10 µl was set up containing: 1 µl of 10x *Taq* DNA Polymerase buffer (Promega) with final concentration of $MgCl_2$ optimized to a range of 2.0-3.5, 0.2 µl dNTP mix (to a final concentration of 800 µM), forward and reverse primer optimized at 0.25-1.0 µM, 0.35 µl Sybr Green 0.1 %, 0.075 µl Taq DNA polymerase (5 U/µl), and 5 µl template DNA synthesized from 0.5-1.0 µg RNA. Six serial dilutions of plasmid DNA diluted 1 in 7 were assembled in duplicates in 72-well reaction plates. 5 ng of linearized plasmid DNA was used in the first standard to calculate for expected number of copies. A no-template control (NTC) was included in the run to test for DNA contamination.

The cycling conditions were optimized for each transcript but default setting was initially used which were as follows: 94ºC initial hold step, followed by 20 seconds each at 94ºC, 60ºC, and 72ºC for 40 cycles. Fluorescence was acquired at the end of each cycle after the extension step to the FAM/SYBR green channel. A hold step at 94ºC for 20 sec was set to denature all annealing pairs before the melt curve analysis was performed at 65ºC rising to 94ºC at 1ºC per second increments.

2.5.2 Optimization of primer concentration

Primer concentration matrix which ranged between 0.1–1.0 µm, were set up using standard curve generated from pDNA (Section 2.3.3.1). A no template control with

the lowest and highest concentration was run alongside to detect contamination and amplification of primer dimers and non-specific products. Each primer combination was set up in duplicates. Six serial dilutions of linearized pDNA with the insert of the gene of interest were included in the run. Combinations of reverse and forward primers that yielded the lowest Cq without primer dimers were selected for each gene.

2.5.3 Magnesium optimization

After the suitable primer concentration for each gene of interest was identified, the amount of $MgCl_2$ required to run an optimized RT-qPCR assay was determined. $MgCl_2$ titration from 1.5–5.0 mM in 0.5 mM increments was set up. Similar standard curves used in primer optimization were set up alongside different concentrations of $MgCl_2$. The assay was run in triplicates and the magnesium concentration that showed the lowest Cq and single melt peak curve with the least background was selected.

2.5.4 Annealing temperature optimization

The optimal annealing temperature for amplification was also determined for each gene after optimising magnesium and primer concentration. The optimized standard curves taken from real time qPCR runs using pDNA samples were used to determine the efficiency of the PCR assays.

2.5.5 Determination of optimum cDNA template concentration

The best concentration of cDNA for RT-qPCR was determined for lymph node and abomasal tissues. This step checks for inhibitory factors in the RT-qPCR assays that

are associated with highly concentrated template. Also, too dilute templates may give rise to low detectability and non-specific amplifications.

A pool of neat cDNA from each of the samples was diluted 1/10 to 1/80 in nuclease-free water to generate standard curves using the optimized primer and magnesium concentration and annealing conditions. Standard curves from serial dilutions of pooled cDNA were used to confirm the efficiency and to determine the working concentration for the assays. The lowest cDNA concentration that gave the lowest Cq value and single peak melt curve was selected.

2.5.6 Selection of reference genes

Real time-qPCR runs on all cDNA samples were performed on four reference genes namely: GAPDH, HPRT, YWHAZ, and SDHA, which had been optimized for use in sheep tissues (Smeed *et al.*, 2007). The stability of each reference gene was evaluated for each RT batch in both lymph node and abomasal tissues (Table 1, Appendix 1). Each sample from one RT reaction was assayed in triplicate. The plasmid dilution series (Section 2.5.1) was used as a standard curve. Two reference genes were selected using GeNorm v3.4 (Vandesompele *et al.*, 2002) and the NormFinder Microsoft Excel applet (Andersen et al., 2004). The algorithms of these applications determined the most suitable gene in the given samples taking into account the average pairwise variation with all other tested reference genes. Optimal normalization gene was identified among other candidate genes that allowed efficient estimation of overall expression and variation between sample subgroups. YWHAZ and SDHA were chosen to normalize the RT-qPCR assays for both lymph node and abomasal tissues.

2.6 Relative and absolute quantification assay

2.6.1 Experimental set-up

For the relative quantification assay, 15 samples were used from a total of 57 animals sourced from the phenotype experiment (Beraldi et al., 2008). This was represented by the five most resistant and five most susceptible animals and the five uninfected controls for all 14 genes. The groupings were defined according to the infection ranks of the lambs based on their FEC and adult worm count (Table 1, Appendix 1).

Absolute quantification assay for five genes, which were found to have significant differential expression based on the relative quantification assay, was used in 55 animals analysed individually and also in four groups. Four groups consisted of 10 uninfected controls and 15 each of the resistant, intermediate, and susceptible animals based on their infection ranks (Table 5.1). Two of the original 57 samples were excluded from the assay due to RNA degradation. Both relative and absolute copy number expression levels were quantified in three replicates in three separate RT-qPCR runs, each time using cDNA from a different RT reaction.

2.6.2 Data processing and analysis

Gene expression levels were calculated in GenEx version 5.3.4.157 (www.multid.se) using the comparative 2-($^{\Delta\Delta}$ Cq) method and normalized to the geometric mean of the stably-expressed reference genes (SDHA and YWHAZ). Fold changes were calculated from delta Cq values using GenEx.

Normalized copy numbers were obtained using the normalization factor determined by GeNorm. The expression levels were normalized by dividing the copy number

derived from the standard curve by the calculated normalization factor for each individual sample. Plasmid serial dilutions were run alongside cDNA samples for each gene to determine the copy numbers for each sample.

The known copy numbers of plasmid DNA were used to estimate copy number of samples based on the formula:

Number of molecules per ng = $\{[1x10^{-9}]/(M\ g/mol)] \times [6.023x10^{23}\ molecules/mol]\}$

Where (M) = size of plasmid and insert size in bp x 660 g/mol per bp

2.6.3 Normalization of real time data

Copy numbers of each gene of interest was normalized to allow for experimental variation in starting material both in the RT step and the cDNA template used in the RT-qPCR assay. This also checks for technical errors within the run. The normalization coefficient of each sample was determined based on the expression of the selected reference genes YWHAZ and SDHA. Cq values of both genes were exported in Excel format, and then the GeNorm software (Vandesompele *et al.*, 2002) was ran to calculate for individual sample normalization coefficient.

2.6.4 Statistical analysis

The mixed effect model of general linear analysis (using Minitab 15.1.0.0) was used to establish common variance among groups. This evaluated normal data distribution and calculated the variance associated with fixed effects of RT-qPCR replicate runs, reverse transcription steps and the effect of individual sheep differences within each group. Statistical differences between groups were determined by one-way ANOVA

with Tukey-Kramer's post-hoc test for multiple pairwise comparison analysis, in GenEx. The correlations were analyzed with Spearman's correlation coefficient using Graphpad Prism v 5.0. P-values less than 0.05 were considered significant.

2.7 Identification of *Ovar DRB1*0203* allele

Identification of allele Ovar *DRB1*0203* was conducted on the same group of 15 animals used in relative quantification assay (Table 5.1). Primers that had been used previously (Schwaiger *et al.*, 1995) were utilized to run a standard PCR as described in Section 2.3.2. 50 ng of genomic DNA extracted from the 15 lambs were used as template (Section 2.8.1.5).

PCR products were purified (Section 2.4.3) and sequenced as described in Section 2.4.5. At least three independent sequences were generated from each sample, which were aligned with *Ovar DRB*0203* allele using ClustalW2 (Larkin *et al.*, 2007) to generate a consensus. Nucleotide consensus sequences were aligned with *Ovar DRB*0203* allele and the archetypal *Ovar DRB1*. Subsequently, the nucleotide sequences were translated to predicted amino acids and compared with the protein of *DRB*1101*.

2.8 Single nucleotide polymorphism analysis of IL21 and IL21R

2.8.1 Generation of full length sequence of IL21 and IL21R

2.8.1.1 Cloning and sequencing of gene fragments

Sets of primers were designed based on the bovine mRNA sequences (Table 6.2), to generate amplicons that span the coding regions of IL-21 and IL-21R. Standard PCR (Section 2.3.2) was performed and the product purified (Section 2.3.4) for

subsequent cloning and sequence analysis as described in Section 2.4. At least three independent sequences were generated from each clone.

2.8.1.2 Rapid amplification of cDNA ends (RACE)

The sequences of the 5'UTR and 3'UTR of bovine IL-21 mRNA were not available in the genetic sequence database (Genbank®). In order to generate a full length sequence spanning the coding region of IL-21, RNA ligase-mediated rapid amplification of 5' and 3' cDNA ends (RLM-RACE) was performed using the Invitrogen GeneRacer™ kit.

2.8.1.2.1 Primer design for RACE

At least two gene-specific primers (GSPs) were designed initially for 5' end of IL-21. The primer sets were designed on the basis of the following criteria: 50-70 % GC content, 23-28 nucleotides long, and an annealing temperature greater than 72ºC to improve the specificity of the PCR.

Four representative RNA samples extracted from the gastric lymph nodes selected for high concentration and purity were used for RLM-RACE. The integrity and quality of RNA has been evaluated previously (Section 2.2.1.3).

2.8.1.2.2 Dephosphorylating RNA

To eliminate truncated mRNA and non-mRNA from subsequent ligation with the GeneRacer™ RNA Oligo, 5' phosphates was removed. Dephosphorylation was set up on ice in a 10 µl volume reaction containing 1 µg of total RNA, 1 µl 10x CIP buffer, 1 µl RNaseOut™ (40 U/ µl), CIP (10 U/ µl), and DEPC water. The reaction was incubated at 50ºC for 1 hr, and then spun briefly before placing on ice.

2.8.1.2.3 Precipitation of RNA

After dephosphorylation, RNA was precipitated by adding 90 µl DEPC and 100 µl phenol:chloroform:isoamyl alcohol, (25:24:1) included in the kit. The mix was vortexed vigorously for 30 sec before centrifuging at 12,060 x g for 5 min. The aqueous top phase was aspirated and transferred to a microcentrifuge tube (~100 µl). A 2 µl volume of 10 mg/ml mussel glycogen and 10 µl 3 M sodium acetate was added into the tube and mixed before freeze drying for 10 min. To pellet RNA, the microtube was centrifuged at 12,000 x g for 20 minutes at 4°C. The supernatant was removed by pipette and to the pellet was added 500 µl 70% ethanol. The mixture was inverted several times, vortexed briefly, and then centrifuged for 2 min at 4°C. Ethanol was removed by pipette and then the remaining pellet was air-dried for 1-2 min at room temperature. The pellet was resuspended in 7 µl nuclease-free water.

2.8.1.2.4 Removing the mRNA cap structure

To remove the 5' cap structure from full-length mRNA, a 10 µl decapping reaction was set up on ice. The 7 µl dephosphorylated RNA was mixed with 1 µl 10x TAP buffer, 1 µl RNaseOut™ (40 U/µl), and 1 µl of TAP (0.5 U/µl). The reaction was incubated at 37ºC for 1 hr, which was followed by a brief centrifugation before placing on ice. RNA was precipitated and pelleted as described in section 2.8.1.2.3 and then re-suspended in 7 µl DEPC water.

2.8.1.2.5 Ligation of the RNA Oligo to Decapped mRNA

After decapping the mRNA, the GeneRacer™ RNA Oligo was ligated into the 5' end of the mRNA. The 7 µl dephosphorylated, decapped RNA was added into the pre-aliquoted, lyophilized GeneRacer™ RNA Oligo (0.25 µg), pipetted several times to

ensure even mixing and resuspension of the RNA Oligo, then centrifuged briefly to collect fluid. The reaction was incubated for 5 min to relax the RNA secondary structure, and then placed on ice for 2 min. The following reagents were then added to the tube: 1 µl each of 10X ligase buffer, 10 mM ATP, RNaseOut™ (40 U/µl), and T4 RNA ligase (5 U/µl). The reaction was incubated at 37ºC for 1 hr followed by brief centrifugation before placing on ice.

2.8.1.2.6 Reverse transcription

RNA was precipitated as described previously (Section 2.8.1.2.3). The RNA pellet was resuspended in 10 µl nuclease-free water then proceeded to reverse transcribing mRNA using SuperScipt III RT kit (Invitrogen). To 10 µl ligated RNA was added 1 µl each of primers in the kit, DNTP mix, and sterile water. The reaction was incubated at 65ºC for 5 min to remove any secondary RNA structure. Subsequent steps were based on the protocol as described in Section 2.2. The reverse transcribed RNA was stored at -20ºC.

2.8.1.3 Amplifying cDNA ends

In order to amplify the cDNA with known priming site at the 5' end, PCR Setup was set up. The reaction consisted of: 4.5 µl GeneRacer™ 5' Primer (10 µM), 1.5 µl reverse GSP (10 µM), RT template, 5 µl 10x Pfx amplification buffer, 1.5 µl dNTP, 0.5 µl Platinum® Pfx DNA Polymerase (2.5 U/µl), 1 µl $MgSO_4$ (50 mM), and nuclease-free water to a final volume of 50 µl. The annealing conditions consisted of: an initial denaturation step at 94ºC for 2 min, followed by further denaturation at 94ºC for 30 sec, annealing at 67ºC for 30 sec and extension at 68ºC for 1 min in 25 cycles. After PCR, the product was analysed by gel electrophoresis (Section 2.3.3).

If no distinct band but a smear was visualized, primers were designed to perform Nested PCR. Nested PCR would increase the specificity and sensitivity of RACE products for the 5' ends of the gene.

2.8.1.3.1 Nested PCR

To perform nested PCR, 1 µl of the original PCR product was used as a template. This was mixed with 1 µl GeneRacer™ 5' Nested (10 µM), Reverse Nested GSP (10 µM), 5 µl 10x PCR buffer, 1 µl dNTP Mix (10 mM each), 1 µl Platinum® Pfx DNA Polymerase (2.5 U/µl), 1 µl $MgSO_4$ (50mM), and sterile water to a final volume of 50 µl. The cycling parameters used to amplify the product were: 94ºC for 2 min, 94ºC for 30 sec, 65ºC for 30 sec, 68ºC for 2 min for 15-25 cycles and a final extension stage at 68ºC for a cycle of 10 min. The nested PCR product was analysed on an agarose gel (Section 2.3.3), then gel-purified (Section 2.3.5) to set up the TOPO® cloning reaction.

2.8.1.4 Cloning and sequencing of RACE products

The TOPO® cloning reaction protocol was applied to the nested PCR product for eventual transformation into competent *E. coli* cells. The reaction consisted of the following: 100 ng of PCR product, 1 µl of salt solution, and sterile water to a total volume of 5 µl before adding 1 µl of TOPO® vector (One Shot® TOP10). The reaction was incubated at 23ºC for 5 min, and then placed on ice.

2.8.1.4.1 Transforming One Shot® TOP10 Competent cells

An aliquot of 2_µl TOPO® cloning reaction was added into a vial of One Shot® chemically-competent *E. coli*. The cells were incubated for 5 min then heat-shocked

for 30 sec at 42ºC. The tubes were transferred on ice before adding 350 µl of SOC medium. The tube was then placed horizontally in a shaker incubator (200 rpm) at 37ºC for one hour. A 10-50 µl volume of each transformant was spread on a pre-warmed selective plate and then incubated overnight at 37ºC. Ten colonies were picked from the plate, followed by overnight incubation in orbital shaker at 37ºC in LB agar containing 50 µg/ml kanamycin. Plasmids were isolated (Section 2.4.3) and then evaluated for concentration and purity (Section 2.2.1.1). Plasmid inserts were analysed by restriction analysis (Section 2.4.4).

2.8.1.5 Genomic DNA extraction from tissues

Genomic DNA of 55 lambs which served as template for SNP analysis was extracted using the DNeasy Blood kit (Qiagen®). Frozen whole blood cells in EDTA (0.5 M, pH 8.0) were thawed completely. To 25 µl of blood cells were added 20 µl of proteinase K (20 mg/ml) and 175 µl PBS. The tube was vortexed to mix thoroughly before adding 200 µl of buffer AL, and then incubated at 56ºC for 10 min. A 200 µl volume of ethanol was added and mixed before loading into DNEasy columns. The tubes were centrifuged at 6,000 x g for 1 min. The flow through was discarded and the spin column was placed into a 1.2 µl collection tube. 500 µl buffer AWI was added and then centrifuged for 1 min at 6,000 x g. The flow through was discarded and the tube placed in a new collection tube. 500 µl of AW2 buffer was added and then centrifuged for 3 min at 20,000 x g. The flow through and collection tube was discarded. Finally, the spin column was placed in a clean microcentrifuge tube. 500 µl of buffer AE was pipetted onto the DNEasy membrane, incubated for 1 min and then centrifuged at 6,000 x g to elute the gDNA.

2.8.2 High-resolution Melt Analysis (HRM)

High resolution melt analysis is a technique that detects variation in nucleic acid sequences after amplification of the gene by PCR (Nguyen-Dumont *et al.*, 2009;Vossen *et al.*, 2009). A specialized dye is used which is highly fluorescent when bound to double stranded DNA and poorly fluorescent when unbound. Post-amplification, the PCR product is gradually denatured by increasing the temperature in small increments. This results in release of the dye and drop in fluorescence producing the characteristic melting profile. HRM characterizes DNA samples according to their dissociation behaviour as they transition from double-stranded to single-stranded DNA (ssDNA) with increasing temperature.

2.8.2.1 Primer design

Specific considerations were made in designing primers for HRM PCR in addition to those mentioned in Section 2.2.3.1. Primers were 18-30 nucleotides long, have melting temperature of ≥ 56ºC, and GC content of 40-60 %.

2.8.2.2 High resolution melt PCR

HRM PCR assays were performed with the Rotor-Gene Q using the Type-it® HRM™ PCR protocol with reactions set up using CAS-1200 Precision Liquid Handling system (all Qiagen). The top ten each of the most resistant and most susceptible lambs based on their infection ranks were used for HRM analysis. The reaction mix was composed of 5.0 µl 2x HRM PCR Master Mix, 0.7-1.0 µl of forward and reverse primer (10 µM), 1-3 ng of gDNA template and sterile water to make a final volume of 10 µl. The recommended cycling protocol for HRM analysis on the RotorGene-Q machine was initially used, and then optimized for each specific

gene fragment of the full-length sequence. Cycling conditions included an initial PCR activation step of 5 min at 95ºC followed by 3-step cycling which consisted a denaturation step of 10 sec at 95ºC and a combined annealing/elongation step of 30 sec at 55ºC for 45 cycles. The amplification step was followed by continuous fluorescence data acquisition of the melting points which ran for 2 sec ramping between 65- 95ºC at 0.1ºC increments.

2.8.2.3 HRM-PCR data analysis

2.8.2.3.1 Product amplification analysis

All HRM-PCR data analyses were done using the Rotorgene-Q software version 2.02 (Corbett Life Science). Prior to HRM data analysis, quantitative real-time amplification of the PCR product for each sample was analysed. A filtering system was instituted to improve the efficiency of the subsequent HRM analysis which included: Cq values of the amplification step at < 30, individual reaction efficiency of > 1.4, and a single melt peak generated by melt curve analysis which is indicative of a single amplified product. Gene fragments that did not meet these criteria were repeated or optimized.

2.8.2.3.2 Normalization of HRM data

HRM analysis was viewed as a normalized or a difference plot. Normalized curves provided differences in genotypes based on curve shifting and curve shape change. Difference plots draw a visual interpretation of melt curve transitions as distinguished change in fluorescence of a sample to a selected control at each temperature transition.

The regions at least 5ºC below and above the Tm of the samples were manually selected for each HRM assay, encompassing representative baseline data for the pre-melt and post-melt phases. Data points within these regions were included in the analysis.

In the difference graph plot, the genotype bar was selected to identify the sample that will be compared to all other samples against. Since no reference sample was included in the assay, samples with replicates that stick together and lie close to the middle of the spectrum were selected. A confidence value which provided an integrity check of auto-called results was set at 90 %. Samples that fell below this threshold were flagged as variation. PCR products from these samples were purified, cloned and sequenced for SNP analysis.

2.8.3 SNP analysis of sequences

Three clones of each gene fragment that showed differential expression in HRM analysis were sequenced on both strands (Section 2.4.5). All sequences were aligned against the generated ovine full length sequence (Section 2.8.1) using ClustalW2 (Larkin *et al.*, 2007) to generate a consensus. Differences between the genomic

sequences were considered to be SNPs when the similar change was established in two or more of the aligned sequences.

Chapter 3 Development of RT-qPCR assay for quantifying ovine gene transcripts

3.1 Introduction

A major hindrance in understanding the immune-pathological basis of ovine diseases is the lack of adequate assays suitable for quantifying gene expression. The advent of functional and capture protein arrays have found their way to the mainstream of protein expression studies but its application is almost exclusive to mouse models and humans. Whilst it is ideal that protein levels are measured to represent gene expression, there is limited availability of antibodies in sheep (Amsen *et al.*, 2009). Moreover, the low expression of certain genes (e.g. many cytokines in tissues) renders the measurement of protein levels impractical.

Quantifying gene expression by transcript measurements is however limited by its possible misrepresentation of the actual protein levels in tissues. Inconsistent protein expression with mRNA transcript levels has been linked to post-transcription and post-translation modifications. For example, IL-4 and IL-10 can be partially degraded at the translation stage (Amsen *et al.*, 2009; Le *et al.*, 1997), or post-transcriptionally with IL-1 and IL-18 (Harness *et al.*, 2001). However, several studies have established correlation between cytokines measured by RT-qPCR and protein levels quantified by enzyme-linked immunosorbent assay (Heid *et al.*, 1996; Overbergh *et al.*, 2003; Overbergh *et al.*, 1994; Whelan *et al.*, 2003).

In order to quantify the expression of gene transcripts that are associated with resistance and susceptibility to *T. circumcincta*, which is the primary aim of this project, I developed an optimized RT-qPCR assay for sheep. RT qPCR is a tool in quantifying gene expression particularly in lowly-expressed transcripts like cytokines (Giulietti *et al.*, 2001). RT-qPCR allows for the quick and direct measurement of

PCR product during the exponential phase of amplification of genes (Overbergh *et al.*, 2003). The dynamic range of quantification, high accuracy, sensitivity and reproducibility offered by RT-qPCR (Gibson *et al.*, 1996; Heid *et al.*, 1996) makes it a reliable choice in gene expression analysis. Cytokine expression can also be evaluated using techniques such as Northern blot and *in situ* hybridization but these are, at best semi-quantitative and require high amounts of RNA which are not often available (Vandenbroucke *et al.*, 2001). This is overcome by RT-qPCR because it can generate accurate and reliable data even with small amounts of template.

The starting material in this project is the extracted RNA from abomasal mucosa and lymph nodes. It is desired whenever possible that samples to be assayed should have undergone similar RNA extraction process prior to all downstream applications. It was therefore necessary that the assay be optimized before utilizing the RNA samples to ensure that the same material is used for all the assays.

This study aimed to develop a workable RT-qPCR assay that would provide an efficient and accurate quantification of ovine transcripts. The approach involved cloning and sequencing of ovine mRNA transcripts for a panel of cytokines and markers that characterize the immune response of sheep to *T. circumcincta* infection. All factors that influence the quantification of gene transcripts with RT-qPCR were optimized like primer and magnesium concentration, and the annealing conditions.

3.2 Primer design and amplification of RT-qPCR product

Primers were designed as described in Section 2.3.1. It was also considered that primers hybridize to about half the 3'end of one exon and to the 5'end of the next exon to avoid co-amplification of genomic DNA. RT-qPCR was performed using the basic thermo-cycling reactions detailed in Section 2.3.2. Temperature gradients and increasing concentrations of $MgCl_2$ were employed to increase detection sensitivity of the RT-qPCR (Bustin, 2004, 2009).

The RT-qPCR protocol was developed for a panel of cytokines and markers that included IL-2, IL-4, IL-7R, IL-17A, IL-21, IL-23A, IL-25, EBI3, and FOXP3. Other transcripts in the panel (IL-6, IL-10, IL-12p40, IFNγ, and TGFβ) including the reference genes SDHA and YWHAZ primers had been designed previously (Gossner *et al.*, 2011b; Smeed *et al.*, 2007) and re-optimized for the current study. Bovine sequences were utilized initially to design primers for ovine transcripts that were not available from published sequence database; these included IL-7R, IL-17A, IL-21, IL-23A, and IL-25 (Table 3.1). Table 3.2 shows the primers used to sequence the genes used for RT-qPCR.

Table 3.1 Primer sets based on *Bos taurus* sequence for amplification of ovine cytokine transcripts by endpoint PCR.

Gene	Accession No.	Primer sequence (5' to 3')	Anneal temp	Primer (nm)	MgCl2 (mM)	Product size (bp)
IL-7R	XM_599818.4	F: TTCCTGGACTGCCAGATTC R: GAGAGAGTGGGACTCAGTCATC	50°C	500	2.0	537
IL-17A	NM_001008412.1	F: CAGCGAGCACAAGTTCATC R: TTGGGGAGTAGGGGTCAG	55°C	500	3.0	538
IL-21	NM_198832.1	F: ACTATGTGAATGACTTGGATCC R: CTAGGACAGATGCTGATGAATC	52°C	500	2.0	311
IL-23A	XM_588269.4	F: CAGCTCTCACAGCAACTCTGC R: GGTCAACATCGTCAGTCAGTCAG	55°C	500	2.0	635
IL-25	XM_605190.2	F: GAGGAGTGGCTGAAGTGGAAC R: CGGTAGAAGACGGTCTGGTTG	55°C	500	3.0	281
FOXP3	NM_001045933.1	F: TCGTGCACCAGCTCTCAAC R: AACTCATCCACGGTCACAAC	52°C	500	4.0	928

Table 3.2. Primers used in RT- qPCR assay.

Gene	Accession number	Primer sequence (5' to 3')	Product size
IL-2	NM_001009806.1	F: CTTCTACATGCCCAAGGTTAACG	128
		R: CCTTGATCTCTCTGGTGTTCAGG	
IL-4	NM_001009313.2	F: AAACGCCGAACATCCTCAC	126
		R: GCCTAAGCTCAATTCCAGTCC	
IL-6	X68723.1	F: TCCAGAACGAGTTTGAGG	236
		R: CATCCGAATAGCTCTCAG	
IL-7R	XM_599818.4	F: CTCCAGGTCTCCTAATGGCA	172
		R: CAAGGAAGTGAGGATGGGCT	
IL-10	U11421	F: CTGTTGACCCAGTCTCTGCT	224
		R: ACCGCCTTGCTCTTGTTT	
IL-12p40	AF004024	F: TCAGACCAGAGCAGTGAGGT	243
		R: GCAGGTGAAGTGTCCAGAAT	
IL-17A	NM_001008412.1	F: GAAGGCCCACCGATTATC	124
		R: GCATTGATACAGCCTGAGTG	
IL-21	NM_198832.1	F: CAGCAAATAATGGAGACAACG	131
		R: CTCATAAGAATCACAAGAAGGACA	

Table 3.2 cont'n

Gene	Accession number	Primer sequence (5' to 3')	Product size
IL-23A	XM_588269.4	F: ACCTGTGAGCCAATGAGTTC	93
		R: GGTCAACATCGTCAGTCAGTC	
IL-25	NM_001195219.1	F: TGGCTGAAGTGGAACAGTG	198
		R: GACACAGTGTGGACACAGGC	
EBI3	EE824867	F: CACATCATTCATTGCCACGTAC	147
		R: GCTGTGATGTTGAGCACATAGG	
FOXP3	NM_001144947.1	F: CTGACAAGGGTTCCTGCTG	212
		R: GAGGGTGGCATAGGTGAAAG	
IFNγ	NM_001009803	F: CTAAGGGTGGGCCTCTTTTC	237
		R: CATCCACCGGAATTTGAATC	
TGFβ	X76916	F: GAACTGCTGTGTTCGTCAGC	170
		R: GGTTGTGCTGGTTGTACAGG	
SDHA*	NM_174178.2	F: ACCTGATGCTTTGTGCTCTGC	126
		R: CCTGGATGGGCTTGGAGTAA	
YWHAZ*	AY970970.1	F:TGTAGGAGCCCGTAGGTCATC	101
		R: TCTCTCTGTATTCTCGAGCCATC	

* reference genes

```
OVINE      ----------------------------------------CTAAGGTGGATGGCATT 17
BOVINE     AATGTGAGTTTCAATCCTGAAAGTTTCCTGGACTGCCAGATTCATAAGGTGGATGGCATT 948
HUMAN      AATGTGAGTTTCAATCCTGAAAGTTTCCTGGACTGCCAGATTCATAGGGTGGATGACATT 1031
MURINE     AATGTGAGTTTCAATCCCGAAAGTTTCCTGGACTGCCAGATTCATGAGGTGAAAGGCGTT 1076
                                                    .*..****.*:*.*.**

OVINE      CAAGCTAGAGATGAAGCAGAAGGCTTCCTGCAAGACCCTCTTTCTCTGCAGCTAGAGGAG 77
BOVINE     CAAGCTAAAGATGAAGCAGAAGGCTTCCTGCAAGACCCTCTTCCTCCACAGCCAGAAGAG 1008
HUMAN      CAAGCTAGAGATGAAGTGGAAGGTTTTCTGCAAGATACGTTTCCTCAGCAACTAGAAGAA 1091
MURINE     GAAGCCAGGGACGAGGTGGAAAGTTTTCTGCCCAATGATCTTCCTGCACAGCCAGAGGAG 1136
            **** *..** **.* .***.* ** ****...*  .  ** **  .**.* ***.**.

OVINE      AGTGAGAAGCAGAGGTTCAGAGGCGGGATGCAGGGTCCCAGCTGGCCCTCCGAGCAAGCA 137
BOVINE     AGTGACAAGCAGAGGTTCAGAGCCGGGATGCAGGGTCCCAGCTGGCCCTCCGAGCAAGCA 1068
HUMAN      TCTGAGAAGCAGAGGCTTGGAGGGGATGTGCAGAGCCCCAACTGCCCATCTGAGGATGTA 1151
MURINE     TTGGAGACACAGGGACACAGAGCCGCTGTACACAGTGCAAACCGCTCGCCTGAGACTTCA 1196
           :  ** *..***.*. : .***  *  .*.** .*  *.*.* *  *  * *** .:  *

OVINE      GGCATTACGCCTAAAATTTTCAGAGGAGAGTCACCATTCAGATGCTTGGCTGGGAATGCC 197
BOVINE     GGCATTACCCCTAAAATTTTCAGAGGAGAGTCACCATTCAGATGCCTGGCTGGGAATGCC 1128
HUMAN      GTCATCACTCCAGAAAGCTTTGGAAGAGATTCATCCCTCACATGCCTGGCTGGGAATGTC 1211
MURINE     GTCAGCCCACCAGAAACAGTTAGAAGAGAGTCACCCTTAAGATGCCTGGCTAGAAATCTG 1256
           * **  .* **:.***   * .**.**** *** *. *.* **** *****.*.***

OVINE      AGTGTGTGTGATGCCCCTGGGCTTCCCTCCTCCAGGTCTCCTAATGGCAGGGATGGTGGC 257
BOVINE     AGTGTGTGTGATGCCCCTGGGCTCCTCTCCTCCAGGTCTCCTAATGGCAGGGAAGGTGGC 1188
HUMAN      AGTGCATGTGACGCCCCTATTCTCTCCTCTTCCAGGTCCCTAGACTGCAGGGAGAGTGGC 1271
MURINE     AGTACCTGCAATGCCCCTCCACTCCTTTCCTCTAGGTCCCCTGACTACAGAGATGGTGAC 1316
           ***.  ** .* ******   **    ** ** ***** * :.*  .***.** .***.*

OVINE      AAGAGCAGACCTCTAGTGTACCAGGACCTGCTCCTCAGACCTGGAACTACAAA----CAG 313
BOVINE     AAGAGCAGACCTCTAGTGTACCAGGACCTGCTCCTCGGACCTGGAACTACAAA----CAG 1244
HUMAN      AAGAATGGGCCTCATGTGTACCAGGACCTCCTGCTTAGCCTTGGGACTACAAA----CAG 1327
MURINE     AGAAATAGGCCTCCTGTGTATCAAGACTTGCTGCCAAACTCTGGAAACACAAATGTCCCT 1376
           *.*. .*.**** :***** **.*** * ** *  ...  ***.*. *****    *.

OVINE      CTCCCTG--TCCCCTTCGTTTCCATTCCAACCCGGAATCCTGACATTAAACCCTGTTGCC 371
BOVINE     CTCCCTG--CCCCCTCCGTTTCCATTCCAACCCGGAATCCTGACATTGAACCCTGTTGCC 1302
HUMAN      CACGCTG--CCCCCTCCATTTTCTCTCCAATCTGGAATCCTGACATTGAACCCAGTTGCT 1385
MURINE     GTCCCTGTCCCTCAACCATTGCCTTTCCAGTCGGGAATCCT------GATACCAGTTTCT 1430
            :* ***   * *.: *.**  *: ****. * ********      .*:.**:*** *

OVINE      CAGGGGCAGCCCATCCTCACTTCCTTGGGATCAAGTCAAGAAGAAGCCTATGTCACCATG 431
BOVINE     CAGGGGCAGCCCATCCTCACTTCCTTGGGATCAAGTCAAGAAGAAGCCTATGTCACCATG 1362
HUMAN      CAGGGTCAGCCCATTCTTACTTCCCTGGGATCAAATCAAGAAGAAGCATATGTCACCATG 1445
MURINE     CAGAGACAGCCCATCTCCACTTCCTCAGTACTGAATCAAGAAGAAGCGTATGTCACCATG 1490
           ***.* ********    ******  .* *  .*.************ ************

OVINE      TCCAGCTTCTACCAAAACCAGTGAGTTGTAAGAAACCCAAGAT---------CAGAACCA 482
BOVINE     TCCAGCTTCTACCAAAACCAATGAGTTGTAAGAAACCCAAGAT---------CAGAACCA 1413
HUMAN      TCCAGCTTCTACCAAAACCAGTGAAGTGTAAGAAACCCAGACTGAACTTACCGTGAGCGA 1505
MURINE     TCTAGTTTTTACCAAAACAAATGAATTATAAGAAAACCCTTCCAT-------CGACAACC 1543
           ** ** ** *********.*.***. *.*******.**.  .           .... .

OVINE      TCATGATGACCGAA---------------------------------------------- 496
BOVINE     TCATGATGAACGAAGATG------ACTGAGTCCCACTCTCTCCCACAGCACAAACAAACA 1467
HUMAN      CAAAGATGATTTAAAAGGGAAGTCTAGAGTTCCTAGTCTCCCTCACAGCACAGAGAAGAC 1565
MURINE     AAATGATCACTGAGATGGAAAGTCTGGAATGCTTGCTCTCCCCCGTAGCTCACAGAAGAG 1603
                 .*:*** *   *.
```

Figure 3.1 Alignment of sheep IL-7R nucleotide sequence with cattle (XM_599818.4), human (NM_002185.2), and mouse (NM_008372.4) sequences; with alignment scores against ovine transcripts of 95 %, 77 %, and 67 % respectively. (*) indicates identical nucleotide bases in all sequences.

```
OVINE      ------------------------------------TGAATTTCTGCCAGCTCCAGAA 22
BOVINE     GTTGATCAGCTGAAAAACTATGTGAATGACTTGGATCCTGAATTTCTGCCAGCTCCAGAA 192
HUMAN      GTTGATCAGCTGAAAAATTATGTGAATGACTTGGTCCCTGAATTTCTGCCAGCTCCAGAA 240
PORCINE    GTTGATCAGCTGAAAAATTATGTTCATGACTTGGACCCTGAATTGCTGCCAGCTCCAGAA 122
MURINE     GTTGAACAGCTGAAAATCTATGAAAATGACTTGGATCCTGAACTTCTATCAGCTCCACAA 227
                                              **** * **. ******** **

OVINE      GATGTAAAGAGACACTGTGAGCGGTCAGCTTTTTCATGTTTTCAGAAGGTTCAACTAAAG 82
BOVINE     GATGTAAAGAGACACTGTGAGCGGTCAGCTTTTTCATGTTTTCAGAAGGTTCAACTAAAG 252
HUMAN      GATGTAGAGACAAACTGTGAGTGGTCAGCTTTTTCCTGCTTTCAGAAGGCCCAACTAAAG 300
PORCINE    GATGTACAGAGACACTGTGAGCAGTCAGCTTTTTCATGTTTTCAGAAGGTCGAACTAAAG 182
MURINE     GATGTAAAGGGGCACTGTGAGCATGCAGCTTTTGCCTGTTTTCAGAAGGCCAAACTCAAG 287
           ****** **. ..******** .  ******** *.** **********   ****.***

OVINE      TCAGCAAATAATGGAGACAACGAAAAGATAATCAACATACTAACTAAACAGCTGAAGAGG 142
BOVINE     TCAGCAAATAATGGAGACAACGAAAAGATAATCAACATATTAACTAAACAGCTGAAGAGG 312
HUMAN      TCAGCAAATACAGGAAACAATGAAAGGATAATCAATGTATCAATTAAAAAGCTGAAGAGG 360
PORCINE    TCAGCAAATACGGGAGACAATGAAAAGATAATCAATGTATTAATAAAACAGCTGAAGAGG 242
MURINE     CCATCAAACCCTGGAAACAATAAGACATTCATCATTGACCTCGTGGCCCAGCTCAGGAGG 347
            ** **** .. ***.**** .*.* .:*.****: .:.  ..  ....**** *.****

OVINE      AAACTGCCTCCCACAAATGCAGGGAGAAGACAGAAACATGAACTAACATGTCCTTCTTGT 202
BOVINE     AAACTACCTGCCACAAATACAGGGAGAAGACAGAAACATGAAGTAACATGTCCTTCTTGT 372
HUMAN      AAACCACCTTCCACAAATGCAGGGAGAAGACAGAAACACAGACTAACATGCCCTTCATGT 420
PORCINE    AAACTACCTCCCACAAATGCAGGGAGAAGACAGAAACATGGGCTAACATGTCCTACATGT 302
MURINE     AGGCTGCCTGCCAGGAGGGGAGGAAAGAAACAGAAGCACATAGCTAAATGCCCTTCCTGT 407
           *..* .*** *** .*. . ***.*..*.******.** . .  :*.*** ***:* ***

OVINE      GATTCTTATGAGAAAAAGCCACCCAAGGAATACCTAGAAAGACTGAAATCGCTCATCCAA 262
BOVINE     GATTCTTATGAGAAAAAACCACCCAAGGAATACCTAGAAAGACTGAAATCACTCATCCAA 432
HUMAN      GATTCTTATGAGAAAAAACCACCCAAAGAATTCCTAGAAAGATTCAAATCACTTCTCCAA 480
PORCINE    GATTCGTATGAGAAAAAACCAATCAAAGAATTCCTAGAAAGACTGAAATCGCTCATCCAA 362
MURINE     GATTCGTATGAGAAAAGGACACCCAAAGAATTCCTAGAAAGACTAAAATGGCTCCTTCAA 467
           ***** **********...**. ***.****:********** * **** .** .* ***

OVINE      AAG---------------------------------------------------------- 265
BOVINE     AAG---------------------------------------------------------- 435
HUMAN      AAGGTATCTACCTTAAGTTTCATTTGATTTTCTGCTTTATCTTTACCTATCCAGATTTGC 540
PORCINE    AAG---------------------------------------------------------- 365
MURINE     AAG---------------------------------------------------------- 470
           ***

OVINE      ---------------------------------AT------------------------- 267
BOVINE     ---------------------------------ATGATTCATCAGCATCTGTCCTAG--- 459
HUMAN      TTCTTAGTTACTCACGGTATACTATTTCCACAGATGATTCATCAGCATCTGTCCTCTAGA 600
PORCINE    ---------------------------------ATGATTCATCAGCATCTGTCCTAG--- 389
MURINE     ---------------------------------ATGATTCATCAGCATCTCTCCTAGAAC 497
                                              **
```

Figure 3.2 Alignment of sheep IL-21 nucleotide sequence with cattle (NM_198832.1), human (NM_001207006.1), pig (NM_214415.1), and mouse (NM_021782.2) sequences; with alignment scores against ovine transcripts of 97 %, 86 %, 91 %, and 72 % respectively. (*) indicates identical nucleotide bases in all sequences.

```
OVINE      ----------------------------------------------------------AT 2
BOVINE     GTCAGAGGACAGCAGCCCTGCTTGGACTCGGGGCCAACAGCTCTCACAGCAACTCTGCAT 131
HUMAN      GCCTGGGGGCAGCAGCCCTGCCTGGACTCAGTGCCAGCAGCTTTCACAGAAGCTCTGCAC 291
PORCINE    GCCTGAGGGCAGCAGCCCTGCTTGGGCTCAAGGCCAGCAGCTCTCACAGCAGCTCTGCAC 277
MURINE     GCCTAGGAGTAGCAGTCCTGACTGGGCTCAGTGCCAGCAGCTCTCTCGGAATCTCTGCAT 237
                                                                     *

OVINE      GCTAGCCTGGAGTGCACACCTACCAATGGGACATGTGGATCTACCAAGAGAAGAAGGAGG 62
BOVINE     GCTGGCCTGGAGTGCACACCTACCAATGGGACATGTGGATCTACCAAGAGAAGAAGGAGG 191
HUMAN      ACTGGCCTGGAGTGCACATCCACTAGTGGGACACATGGATCT---AAGAGAAGAGGGAGA 348
PORCINE    GCTGGCCTGGACTGCACATCTACCAATGGGACATGTGGATCTACCAAGAGAAGAGGGAGA 337
MURINE     GCTAGCCTGGAACGCACATGCACCAGCGGGACATATGAATCTACTAAGAGAAGAAGAGGA 297
           .**.*******  *****   ** *. ****** .**.****   *********.*..*.

OVINE      TGATGAGACTACAGATGATGTCCCCCGTATCCAGTGTGAGGATGGCTGTGATCCACAAGG 122
BOVINE     TGATAAGACTACAGATGATGTCCCCCGTATCCAGTGTGAGGATGGCTGTGATCCACAAGG 251
HUMAN      TGAAGAGACTACAAATGATGTTCCCCATATCCAGTGTGGAGATGGCTGTGACCCCCAAGG 408
PORCINE    TGATGAGACTACAAGTGAAGTCCCCCATATCCAGTGCGGGGATGGCTGTGATCCTCAGGG 397
MURINE     TGAAGAGACTAAAAATAATGTGCCCCGTATCCAGTGTGAAGATGGTTGTGACCCACAAGG 357
           ***:.******.*..*.*:** ****.********* *..***** ***** ** **.**

OVINE      ACTCAGGGACAACAGTCAGTCCTGCTTGCGAAGGATTCATCGAGGCCTGGTTTTTTACGA 182
BOVINE     ACTCAGGGACAACAGTCAGTCCTGCTTGCAAAGAATTCATCGAGGCCTGGTTTTTTACGA 311
HUMAN      ACTCAGGGACAACAGTCAGTTCTGCTTGCAAAGGATCCACCAGGGTCTGATTTTTTATGA 468
PORCINE    ACTCAGGGACAACAGTCAGTCCTGCTTGCAAAGGATCCACCAAGGCCTGGTTTTTTATGA 457
MURINE     ACTCAAGGACAACAGCCAGTTCTGCTTGCAAAGGATCCGCCAAGGTCTGGCTTTTTATAA 417
           *****.********* **** ********.***.** *. *..** ***. ****** .*

OVINE      GAAGCTTCTGGGCTCAGATAGTTTCACAGGGGAGCCTTCTCTATTCCCAGATGGCCCTGT 242
BOVINE     GAAGCTGCTGGGCTCAGATATTTTCACAGGGGAGCCTTCTCTACTCCCAAATGGCCCTGT 371
HUMAN      GAAGCTGCTAGGATCGGATATTTTCACAGGGGAGCCTTCTCTGCTCCCTGATAGCCCTGT 528
PORCINE    GAAGCTGCTGGGCTCAGACATTTTCACAGGGGAGCCTTCTCTACACCCTGATGGCTCTGT 517
MURINE     GCACCTGCTTGACTCTGACATCTTCAAAGGGGAGCCTGCTCTACTCCCTGATAGCCCCAT 477
           *.* ** ** *..** ** *  ****.********** ****. :***:.**.** * .*

OVINE      GGACCAGCTTCACGCCTCCATACTGGGCCTCAGGGAACTCTTGCAGCCCAAGGGTCACCA 302
BOVINE     GGACCAGCTTCACGCCTCCATACTGGGCCTCAGGGAACTCTTGCAGCCCAAGGGTCACCA 431
HUMAN      GGGCCAGCTTCATGCCTCCCTACTGGGCCTCAGCCAACTCCTGCAGCCTGAGGGTCACCA 588
PORCINE    GGGCCAGCTTCACGCCTCCCTACTGGGCCTCAGGCAACTCTTGCAGCCCGAGGGTCACCA 577
MURINE     GGAGCAACTTCACACCTCCCTACTAGGACTCAGCCAACTCCTCCAGCCAGAGGATCACCC 537
           **. **.***** .*****.****.**.*****  ***** * ***** .***.*****.

OVINE      CTGGGAAGCTGAGCAGACTCCAAGCCCTATTCCCAGCCAGCCATGGCAGCGCCTCCTTCT 362
BOVINE     CTGGGAAACTGAGCAGACTCCAAGCCCTATTCCCAGCCAGCCATGGCAGCGCCTCCTTCT 491
HUMAN      CTGGGAGACTCAGCAGATTCCAAGCCTCAGTCCCAGCCAGCCATGGCAGCGTCTCCTTCT 648
PORCINE    CTGGGAGACTGAGCAGACGCCAAGCCCCAGTCCCAGCCAGCCCTGGCAACGCCTCCTTCT 637
MURINE     CCGGGAGACCCAACAGATGCCCAGCCTGAGTTCTAGTCAGCAGTGGCAGCGCCCCCTTCT 597
           * ****..*  *.****  **.****  * * * ** ****. *****.** * ******

OVINE      CCGTCTCAAGATCCTTCGAAGCCTCCAGGCCTTTGTGGCTGTAGCTGCCCGGGTCTTTGC 422
BOVINE     CCGTCTCAAGATCCTTCGAAGCCTCCAGGCCTTTGTGGCTGTAGCTGCCCGGGTCTTTGC 551
HUMAN      CCGCTTCAAAATCCTTCGCAGCCTCCAGGCCTTTGTGGCTGTAGCCGCCCGGGTCTTTGC 708
PORCINE    CCGCCTCAAGATCCTTCGCAGCCTCCAGGCCTTTGTGGCTGTAGCTGCCCGGGTCTTCGC 697
MURINE     CCGTTCCAAGATCCTTCGAAGCCTCCAGGCCTTTTTGGCCATAGCTGCCCGGGTCTTTGC 657
           ***   ***.********.*************** **** .**** *********** **
```

Figure 3.3 Alignment of sheep IL-23A nucleotide sequence with cattle (XM_588269.4), human (NM_016584.2), pig (AB521204.1), and mouse (NM_031252.2) sequences; with alignment scores against ovine transcripts of 97 %, 83 %, 87 %, and 75 % respectively. (*) indicates identical nucleotide bases in all sequences.

```
OVINE      CCACGGAGCAGCAACTCT----GAGCCCCTAAAGCCAGCAGCTTAAGGATGACAC----- 473
BOVINE     CCACGGAGCAGCAACTCT----GAGCCCCTAAAGCCAGTAGCTTAAGGATGACAC----- 602
HUMAN      CCATGGAGCAGCAACCCT----GAGTCCCTAAAGGCAGCAGCTCAAGGATGGCAC----- 759
PORCINE    CCATGGAGCAGCAACTCT----GAGCCAGTAAAGCCAGCAGCTTAAGGATGACAC----- 748
MURINE     CCACGGAGCAGCAACTCTGACTGAGCCCTTAGTGCCAACAGCTTAAGGATGCCCAGGTTC 717
           *** ********** **    *** *. **.:* **. **** ******* *..

OVINE      ------------------------------------------------------------
BOVINE     ------------------------------------------------------------
HUMAN      ------------------------------------------------------------
PORCINE    ------------------------------------------------------------
MURINE     CCATGGCTACCATGATAAGACTAATCTATCAGCCCAGACATCTACCAGTTAATTAACCCA 777


OVINE      ------------------------------------------CCAGACCTCCATGG-- 487
BOVINE     ------------------------------------------CCAGACCTCCGTGG-- 616
HUMAN      ------------------------------------------TCAGATCTCCATGG-- 773
PORCINE    ------------------------------------------CAAGACCTCCATTG-- 762
MURINE     TTAGGACTTGTGCTGTTCTTGTTTTGTTTGTTTTGCGTGAAGGGCAAGGACACCATTATT 837
                                                     .**. *:**.* .

OVINE      ------------------CTCAGTAATG------------------------------ 497
BOVINE     ------------------CTCAGTAATG------------------------------ 626
HUMAN      ------------------CCCAGCAAGG------------------------------ 783
PORCINE    ------------------CTCAGCAATG------------------------------ 772
MURINE     AAAGAGAAAAGAAACAAACCCCAGAGCAGGCAGCTGGCTAGAGAAAGGAGCTGGAGAAGA 897
                       * *** .. *

OVINE      ------------------------------------------------------------
BOVINE     ------------------------------------------------------------
HUMAN      ------------------------------------------------------------
PORCINE    ------------------------------------------------------------
MURINE     AGAATAAAGTCTCGAGCCCTTGGCCTTGGAAGCGGGCAAGCAGCTGCGTGGCCTGAGGGG 957


OVINE      ----------------------TTAAGATCAATCTATCAACCTAGACACCTGTGAGCCA 534
BOVINE     ----------------------CTAAGATCAATCTATCAAC-TAGACACCTGTGAGCCA 662
HUMAN      ----------------------CCAAGATAAATCTACCACCCCAGGCACCTGTGAGCCA 820
PORCINE    ----------------------CTAAAATGAAG----------------CTATGAGCCA 793
MURINE     AAGGGGGCGGTGGCATCGAGAAACTGTGAGAAAACCCAGAGCATCAGAAAAAGTGAGCCC 1017
                               .:.*  **                .:.******.

OVINE      A--------------------------TGAGTTCATCGGTCCATTA-ATTTTAATGAG- 565
BOVINE     A--------------------------CAAGTTCATTGGTCCATTA-ATTTTAATGAG- 693
HUMAN      A--------------------------CAGGTTAATTAGTCCATTA-ATTTTAGTGGG- 851
PORCINE    A--------------------------CAAGTTCATCAATCCATTA-ATTTTAATGGG- 824
MURINE     AGGCTTTGGCCATTATCTGTAAGAAAAACAAGAAAAGGGGAACATTATACTTTCCTGGGT 1077
           *                          ..*::.*  ..:.***** * ***. **.*

OVINE      ------------------------------------------------------ACTTATTC 573
BOVINE     ------------------------------------------------------ACTTATTC 701
HUMAN      ------------------------------------------------------ACCTGCAT 859
PORCINE    ------------------------------------------------------ACTTGCTC 832
MURINE     GGCTCAGGGAAATGTGCAGATGCACAGTACTCCAGACAGCAGCTCTGTACCTGCCTGCTC 1137
                                                                .* *. :

OVINE      TG---------------------------------TTGAAAAATTACCAAAA------ 592
BOVINE     TG---------------------------------TTGAAAAATTACCAAAACTGACT 726
HUMAN      AT---------------------------------GTTGAAAATTACCAATACTGACT 884
PORCINE    TG---------------------------------TTGAAAACTTACCTACACTGACT 857
MURINE     TGTCCCTCAGTTCTAACAGAATCTAGTCACTAAGAACTAACAGGACTACCAATACGAACT 1197
         :                                   :  *... ****:* *
```

Figure 3.3 cont'n. Alignment of sheep IL-23A nucleotide sequence with related species.

```
OVINE      ----------------------------------------AGTGTGCTCATGCCTC 16
BOVINE     CCAGCAAAGGACAGAATTCCACTGAGGAGTGGCTGAAGTGGAACAGTGTGCTCATGCCTC 232
HUMAN      CCAGCAAAGGGCAGGACACCTCTGAGGAGCTGCTGAGGTGGAGCACTGTGCCTGTGCCTC 133
PORCINE    CCAGCAAAGGACAGAACCCCATAGAAGAGTGGCTGAAGCAGAATGCTGTGCTCATGCCTC 148
MURINE     CCAGCAAAGAGCAAGAACCCCCGGAGGAGTGGCTGAAGTGGAGCTCTGCATCTGTGTCCC 233
                                                      ** .   .** * *

OVINE      CCCCAGAGACCACCAGCCTCGCCCACCACTCAGAATCCTGCAGTTCCAGCAAGGATGGAC 76
BOVINE     CCCCAGAGACCACCAGCCTCGCCCACCACCCAGAACCCTGCAGTTCCAGCAGGGATGGAC 292
HUMAN      CCCTAGAGCCTGCTAGGCCCAACCGCCACCCAGAGTCCTGTAGGGCCAGTGAAGATGGAC 193
PORCINE    CTCTGGAGATGGCCAGCCCCACTCCCCACCCAGAATCCTGCAAGGCGAGTGAAGACGGAC 208
MURINE     CCCCAGAGCCTCTGAGCCACACCCACCACGCAGAATCCTGCAGGGCCAGCAAGGATGGCC 293
           * * .***.     ** * *.. * **** ****. **** *.  * ** ...** **.*

OVINE      CCCTCAACAGCCGTTCCATCGCCCCCTGGAGATATGAGTTGGACAGAGACTTGAACCGGC 136
BOVINE     CCCTCAACAGCCGCTCCATCGCCCCCTGGAGATATGAGTTGGACAGAGACTTGAACCGGC 352
HUMAN      CCCTCAACAGCAGGGCCATCTCCCCCTGGAGATATGAGTTGGACAGAGACTTGAACCGGC 253
PORCINE    CCCTCAACAGCAGATCCATTGCCCCTTGGAGATACGAGTTGGACAGAGACTTGAACCGGC 268
MURINE     CCCTCAACAGCAGGGCCATCTCTCCTTGGAGCTATGAGTTGGACAGGGACTTGAATCGGG 353
           ***********.*  ****  * ** *****.** ***********.******** ***

OVINE      TCCCGCAGGATCTGTACCACGCACGCTGCCTGTGTCCACACTGTGTCAGCCTCCAGACGG 196
BOVINE     TCCCGCAGGATCTGTACCACGCACGTTGCCTGTGTCCACACTGTGTCAGCCTGCAGACGG 412
HUMAN      TCCCCCAGGACCTGTACCACGCCCGTTGCCTGTGCCCGCACTGCGTCAGCCTACAGACAG 313
PORCINE    TGCCCCAGGACTTGTACCACGCTCGTTGCCTGTGTCCACACTGTGTCAGCCTCCGGACGG 328
MURINE     TCCCCCAGGACCTGTACCACGCTCGATGCCTGTGCCCACACTGCGTCAGCCTACAGACAG 413
                * ** *****  ********** ** ******** **.***** ******** *.***.*

OVINE      GCTCCCACATGGACCCCCTGGGAAACTCAGAGCTGCTCTACCA----------------- 239
BOVINE     GCTCCCACATGGACCCCCTGGGAAACTCAGAGCTGCTCTACCACAACCAGACCGTCTTCT 472
HUMAN      GCTCCCACATGGACCCCCGGGGCAACTCGGAGCTGCTCTACCACAACCAGACTGTCTTCT 373
PORCINE    GTTCCCACATGGATCCCCTGGGTAACTCAGAGCTGCTGTACCACAACCAGACCGTCTTCT 388
MURINE     GCTCCCACATGGACCCGCTGGGCAACTCCGTCCCACTTTACCACAACCAGACGGTCTTCT 473
           * *********** ** * *** ***** *: * .** *****
```

Figure 3.4 Alignment of sheep IL-25 nucleotide sequence with cattle (XM_605190.2), human (AF458059.1), pig (EF584511.1), and mouse (NM_080729.3) sequences; with alignment scores against ovine transcripts of 97 %, 84 %, 84 %, and 81 % respectively. (*) indicates identical nucleotide bases in all sequences.

```
OVINE      ------------------------------TCTACAGTGAACTGGAAGGAGCTACCAT 28
BOVINE     GGTCCATCTCACAGCGAGCACAAGTTCATCTGTCTACAGTGAACTGGAAGGAACTACCAT 60
HUMAN      ------------GCAGGCACAAACTCATCCATCCCCAGTTGATTGGAAGAAACAACGAT 47
PORCINE    --TCCATCTCTCAGCAAGCTCCAGCTCATCCATCTGCAGTACATCAGGAGAAACTACGAT 58
MURINE     -ATCCACCTCACACGAGGCACAAGTGCACCCAGCACCAGCTGATCAGGACGCGCAAACAT 59
                                          *  ***   *  .*.* ...*:*. **

OVINE      GGCGTCTATGAGAACTGCCTCTATGTCACTGCTACT------GCTTCTGAGTCTGGTGGC 82
BOVINE     GGCTTCTATGAGAACTTCATCTATGTCACTGCTACT------GCTTCTGAGTCTGGTGGC 114
HUMAN      GACTCCTGGGAAGACCTCATTGGTGTCACTGCTACT------GCTGCTGAGCCTGGAGGC 101
PORCINE    GACTCCTGTGAGATCCTCGTCCCTGTCACTGCTGCT------TCTGCTGAGCCTGGTGGC 112
MURINE     GAGTCCAGGGAGAGCTTCATCTGTGTCTCTGATGCTGTTGCTGCTGCTGAGCCTGGCGGC 119
           *.   *:. **.. *  * *   ****:***.*.**       ** ***** **** ***

OVINE      TCTTGTGAAGGCAGGGGTCATCATCCCACAGAGTCCAGGCTGCCCACCTACTGAGGACAA 142
BOVINE     TCTTGTGAAGGCAGGAGTCATCATCCCACAGAGTCCAGGCTGCCCACCTACTGAGGACAA 174
HUMAN      CATAGTGAAGGCAGGAATCACAATCCCACGAAATCCAGGATGCCCAAATTCTGAGGACAA 161
PORCINE    TCTCGTGAAGGCGGGAATCATGATCCCACAAAGTCCAGGATGCCCAAAAACTGAGGACAA 172
MURINE     TACAGTGAAGGCAGCAGCGATCATCCCTCAAAGCTCAGCGTGTCCAAACACTGAGGCCAA 179
            .  ********.* ..  *  *****:*..*.  ***  ** ***.. :******.***

OVINE      GAACTTCCCACAGCATGTGAGGGTCAACCTGAACATCGTTAAC---CGGAACACGAACTC 199
BOVINE     GAACTTCCCACAGCATGTGAGGGTCAACCTAAACATCGTTAAC---CGGAGCACAAACTC 231
HUMAN      GAACTTCCCCCGGACTGTGATGGTCAACCTGAACATCCATAAC---CGGAATACCAATAC 218
PORCINE    GAACTTCCCTCAGCATGTAAGGGTCAACTTGAACATCTTGAAC---CGGAGCACACCTGC 229
MURINE     GGACTTCCTCCAGAATGTGAAGGTCAACCTCAAAGTCTTTAACTCCCTTGGCGCAAAAGT 239
           *.******  *.*..***.* ******* * **..** : ***   *  .. .* ..

OVINE      CAG------AAGGCCCACCGATTATCACAAGCGCTCCACCTCACCTTGGACCCTCCACCG 253
BOVINE     CAG------AAGGCCCACCGATTATCACAAGCGCTCCACCTCACCTTGGACTCTCCACCG 285
HUMAN      CAATCCCAAAAGGTCCTCAGATTACTACAACCGATCCACCTCACCTTGGAATCTCCACCG 278
PORCINE    CAG------ACGGCCCTCAGATTACTCCAAACGCTTCACCTCACCATGGACTCTCCAACG 283
MURINE     GAGCTCCAGAAGGCCCTCAGACTACCTCAACCGTTCCACGTCACCCTGGACTCTCCACCG 299
            *.      *.** **:*.** **   *** ** * *** ***** ****. *****.**

OVINE      CAATGAGGACCCTGAGAGGTACCCCTCTGTGATCTGGGAGGCCAAGTGCAGCCACTCAGG 313
BOVINE     CAATGAGGACCCTGAGAGGTACCCCTCTGTGATCTGGGAGGCCAAGTGCAGCCACTCAGG 345
HUMAN      CAATGAGGACCCTGAGAGATATCCCTCTGTGATCTGGGAGGCAAAGTGCCGCCACTTGGG 338
PORCINE    CAACGAGGACCCCGAGAGGTACTCCTCCGTGATCTGGGAGGCCAAGTGCAGCCACTCGGG 343
MURINE     CAATGAAGACCCTGATAGATATCCCTCTGTGATCTGGGAAGCTCAGTGCCGCCACCAGCG 359
           *** **.***** ** **.**  **** **********.** .*****.*****  . *

OVINE      CTGTATCAATGCTGAAGGGAAGGTGGACCACCACATGAACTCTGTCACCATCCAGCAAGA 373
BOVINE     CTGTATCAATGCTGAAGGGAAGGTGGACCATCACATGAACTCTGTCACCATCCAGCAAGA 405
HUMAN      CTGCATCAACGCTGATGGGAACGTGGACTACCACATGAACTCTGTCCCCATCCAGCAAGA 398
PORCINE    CTGTATCAATGCTGAAGGGAAGGAAGATCATCACATGAACTCTGTCCCCATCCAGCAAGA 403
MURINE     CTGTGTCAATGCGGAGGGAAAGCTGGACCACCACATGAATTCTGTTCTCATCCAGCAAGA 419
           *** .**** ** ** **.**  :.**  * ******** ***** . ************

OVINE      GATCCTGGTCCTTCGAAGGGAGTCTCAGCACTGCCCTCACTCCTTCCGGCTGGAGAAGAT 433
BOVINE     GATCCTGGTCCTCCGAAGGGAGTCTCAGCACTGCCCTCACTCCTTCCGGCTGGAGAAGAT 465
HUMAN      GATCCTGGTCCTGCGCAGGGAGCCTCCACACTGCCCCAACTCCTTCCGGCTGGAGAAGAT 458
PORCINE    GATCTTGGTCCTGCGAAGGGAGCCTCGCCACTGCCCCAACTCCTTCCGGCTGGAGAAAGT 463
MURINE     GATCCTGGTCCTGAAGAGGGAGCCTGAGAGCTGCCCCTTCACTTTCAGGGTCGAGAAGAT 479
           **** ******* .. ****** **   ..******  :*:* ***.** * *****..*
```

Figure 3.5 Alignment of sheep IL-17A nucleotide sequence with cattle (NM_001008412.1), human (NM_002190.2), pig (NM_001005729.1), and mouse (NM_010552.3) sequences; with alignment scores against ovine transcripts of 97 %, 83 %, 82 %, and 74 % respectively. (*) indicates identical nucleotide bases in all sequences.

```
OVINE      GCTGGTGGCCGTGGGCTGCACCTGTGTCACCCCCATTGTCCGCCATGTGGCTTAA---GA 490
BOVINE     GCTGGTGGCCGTGGGCTGCACCTGCGTCACCCCCATTGTCCGCCATTTGGCTTAA---GA 522
HUMAN      ACTGGTGTCCGTGGGCTGCACCTGTGTCACCCCGATTGTCCACCATGTGGCCTAA---GA 515
PORCINE    GATGGTGACAGTGGGCTGCACCTGTGTCACCCCCATCGTCCGCCATATTTCTTAA---GA 520
MURINE     GCTGGTGGGTGTGGGCTGCACCTGCGTGGCCTCGATTGTCCGCCAGGCAGCCTAAACAGA 539
           ..*****   ************** ** .** * ** ****.***     * ***   **

OVINE      GCTTTCTGC-------------------------------------------------- 499
BOVINE     GCTTTCTGCCTGACCCCTAC---TCCCCAAATTAGTTAGGTTTCCTGGGGAGTAGACCCA 579
HUMAN      GCTCTGGGGAG--CCCACAC---TCCCCAAAGCAGTTAGACT--ATGGAGAGCCGACCCA 568
PORCINE    GCTTCTAGTCTGACCCCTGC---TCCCCAAATCAGTTAGGCTTTCAGGGGAGTAGACCCA 577
MURINE     GACCCGCGGCTGACCCCTAAGAAACCCCCACGTTTCTCAGCAAACTTACTTGCATTTTTA 599
                *.     *
```

Figure 3.5 cont'n. Alignment of sheep IL-17A nucleotide sequence with related species.

3.3 Sequence verification of ovine transcripts

PCR products were cloned and sequenced to generate plasmids for optimizing the real time RT-qPCR assay. The predicted sequences of sheep that were derived from bovine sequences were aligned with the expected sequences in related species (Figures 3.1-5) using ClustalW2 (Larkin *et al.*, 2007). These sequences formed part of the partial and full length sequences of IL-21and IL-23A, and the two variants of IL-25 produced by our research group (Gossner *et al.*, 2011a). Details on the analyses of variants of IL25 sequences are fully illustrated in Figure 1, Appendix 1. The genomic organization of these variants are also shown in Figure 2, Appendix 1.

3.4 Primer optimization

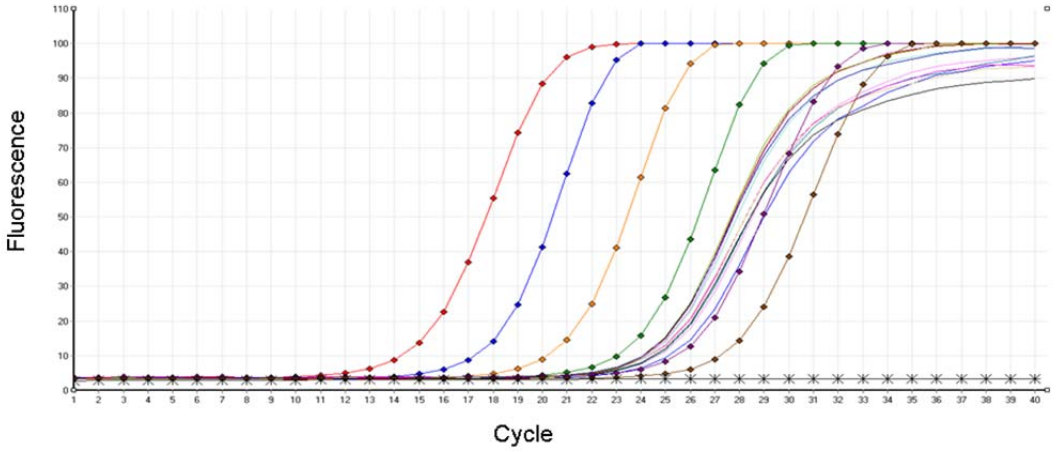
All relevant aspects that could potentially influence the successful run of RT-qPCR assay were considered in developing the assay. A summary on the primer and magnesium concentration, including the annealing temperature that worked optimally for each gene is presented in Table 3.3.

Determination of the workable primer concentration for RT-qPCR is required as this component's limited or excessive availability in the reaction affects not only the product yield but also primer dimer formation (Bustin, 2004; Nolan *et al.*, 2006). Lower primer concentration promotes specificity of the PCR and hence is preferred over a higher concentration without compromise to the efficiency of the reaction.

To determine the best primer concentration for each gene, 100-1000 nM primer concentrations between forward and reverse primers were set up initially (Figure 3.6A). A no-template control was included in the matrix to check for primer dimer or non-specific amplification. A standard curve, made from six serial dilutions of linearized

plasmids with the insert of the target gene was also set up. Any five concentrations with the lowest Cq were included in the second and final run where the concentration with the lowest Cq was selected (Figure 3.6B). In cases where different concentrations had similar Cqs, the lower concentration was selected. Figure 3.6 shows the standard curve generated with optimized primer concentration. The optimized primer concentration for each gene of interest is shown in Table 3.3.

A. Initial primer concentration optimization with standard curve



B. Final primer concentration optimization with standard

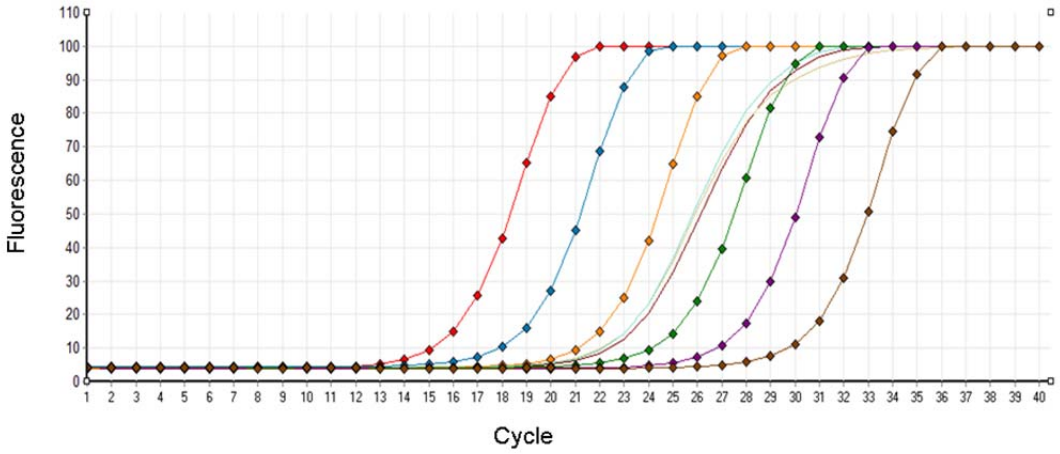


Figure 3.6 Optimization of primer concentration. Example of how the primer concentration was optimized for all transcripts. Initial optimization run included primer concentrations (A) which ranged from 100–900 nm (fine lines) plotted against a standard curve (lines with dots). From this run, five concentrations were selected in the final run (B); from which the concentration that obtained the lowest Cq and corresponding single peak melt curve was selected.

3.5 Magnesium optimization

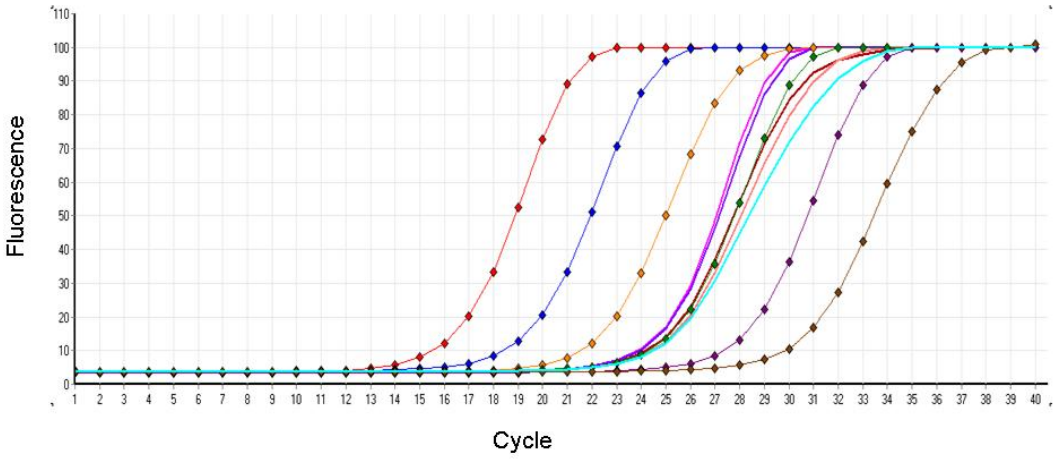
Magnesium is an essential component of PCR as it promotes the activity of the Taq polymerase (Bustin, 2004). High magnesium concentration results to greater product yield but compromises the fidelity of enzymes, thereby causing non-specific amplification. On the other hand, low concentration of this component increases the specificity of the reaction but the quantity of the PCR product becomes reduced. It is therefore paramount to optimize the magnesium concentration of the RT-qPCR to ensure the sensitivity, specificity and reproducibility of the assay.

Each primer pair and template has a different optimal magnesium concentration. A standard curve made from six serial dilutions of pDNA containing the gene of interest was set up together with increasing $MgCl_2$ concentrations that ranged from 2.0-5.0 mM. Figure 3.7 illustrates the difference in the magnesium concentration required for an optimized RT-qPCR in a particular gene. The ideal $MgCl_2$ concentration was the concentration with the lowest Cq and with amplicon-specific product as revealed in the melt curve (not shown).

3.6 Temperature optimization

The annealing temperature and time settings could affect successful priming and amplification of product. Optimum temperature allows primer to anneal efficiently to their targets, preventing non-specific annealing and primer-dimer formation (Bustin, 2004; Taylor *et al.*, 2010). Too low temperature could lead to production of products other than the target as a result of mispriming. Similarly, too high temperature results in inefficient priming. The optimized annealing temperature for each gene is shown in Table 3.3.

A. Optimized Mg concentration of IL7R at low concentration



B. Optimized Mg concentration of IL23A at high concentration

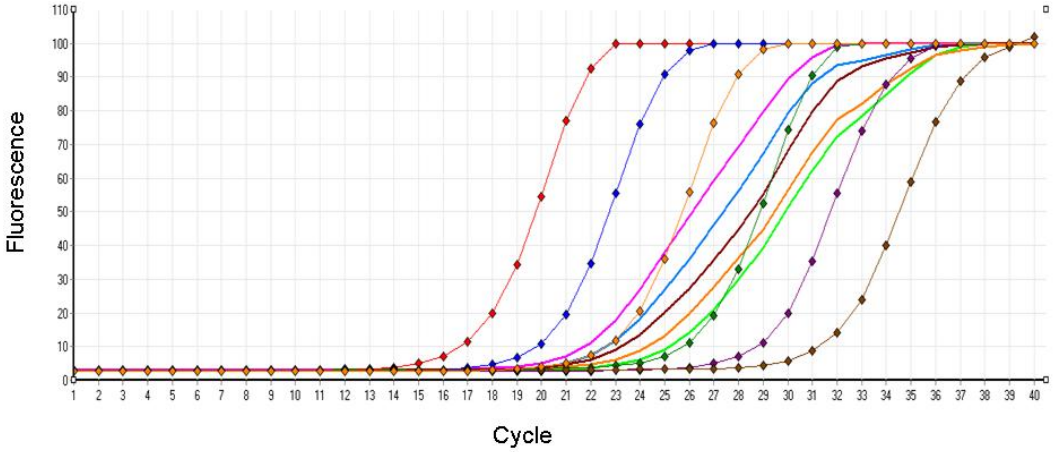


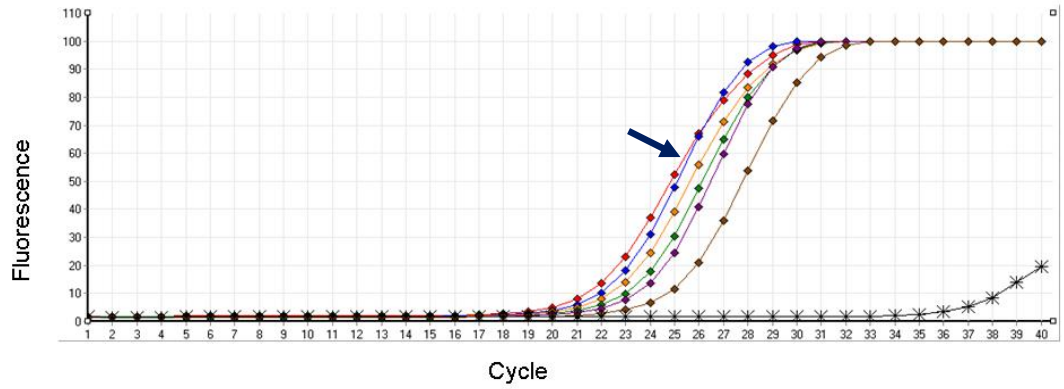
Figure 3.7 Optimization of magnesium concentration. Example of different magnesium concentration optimum for a particular transcript. Standard curve from pDNA (lines with dots) alongside different concentrations of $MgCl_2$ (thick lines). In IL-7R (A), the lowest Cq is with the lowest magnesium concentration of 2 mM (pink) and 2.5 mM (purple). Higher concentration at 4 mM (light blue) is not optimum. IL-23A (B) works best at 3 mM (pink) and poorly at 2 mM (green).

3.7 cDNA dilution optimization

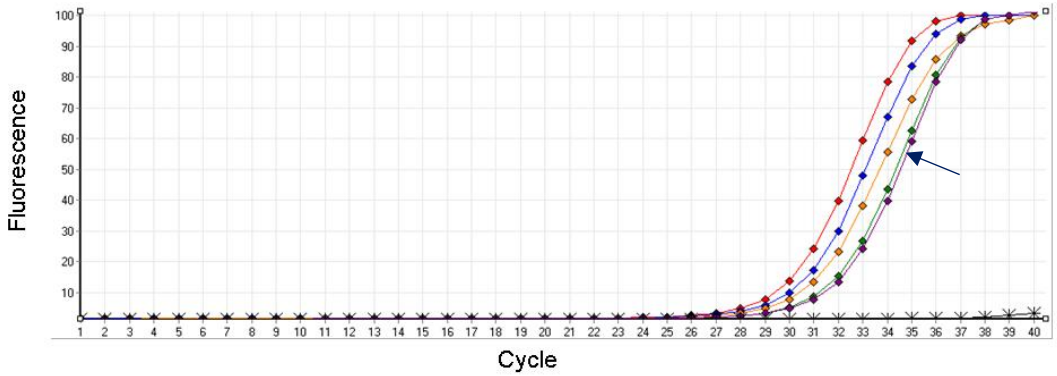
The use of linearized pDNA in constructing standard curves allows repetitive assays inherent in the optimization step. Running the assay with cDNA samples may limit the repetition step as there is usually limited source of the RNA. Generating a standard curve from serial dilutions of cDNA will provide the true efficiency of RT-qPCR runs with the cDNA samples under question. This step also ensures that the concentration range used matches the expected concentration of the unknown sample.

To optimize the cDNA sample dilution for the RT-qPCR assay, a range of dilutions for each gene in both lymph node and abomasum were set up (Figures 3.8 A &B). This is important especially if there is a limited cDNA material available. For example, 1:40 dilution of a template cDNA may be used over 1:10 without compromising the efficiency of the PCR run. Subsequently, the selected concentration (on the basis of Cq and melt curve) of cDNA was run alongside standard curve from pDNA to validate its expected expression within the quantifiable range (Figure 3.8C).

A. Inhibited cycling conditions in concentrated cDNA of YWHAZ in ABM



B. Similar expression levels with different cDNA dilution of SDHA in ALN



C. Optimum cDNA dilution of TGFB1 within quantifiable range

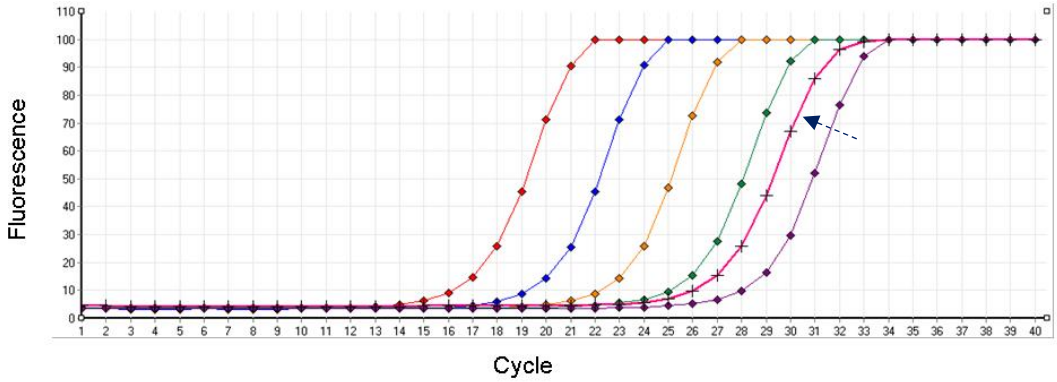


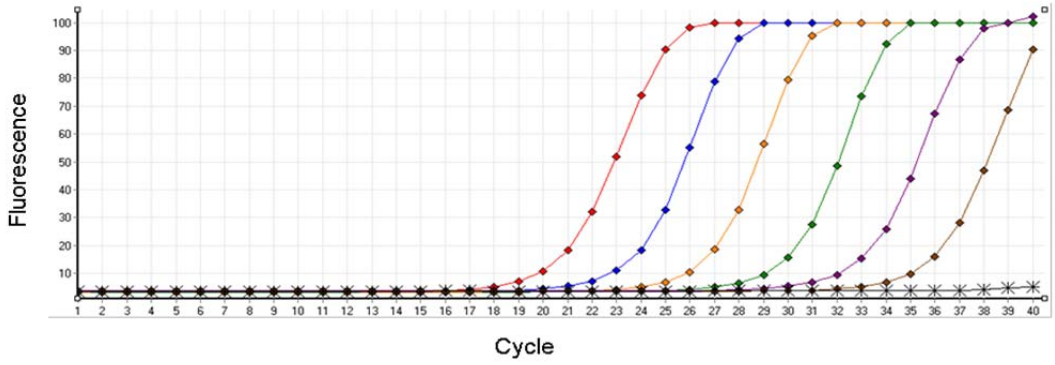
Figure 3.8 cDNA dilution optimization A standard curve was constructed for each gene using serial dilutions of pooled cDNA from lymph node and abomasum. Inhibition could be evident in too concentrated template cDNA (A) as pointed in thick arrow, or vaguely quantifiable after a certain point of dilution (B) in thin arrows, as they stick together even with different concentrations. Selected dilution of a gene (dashed arrow) with the standard curve (C) showing its expression within quantifiable range. Lines marked with stars are no-template controls. and lines with cross points represent a cDNA dilution.

3.8 Construction of standard curves from pDNA

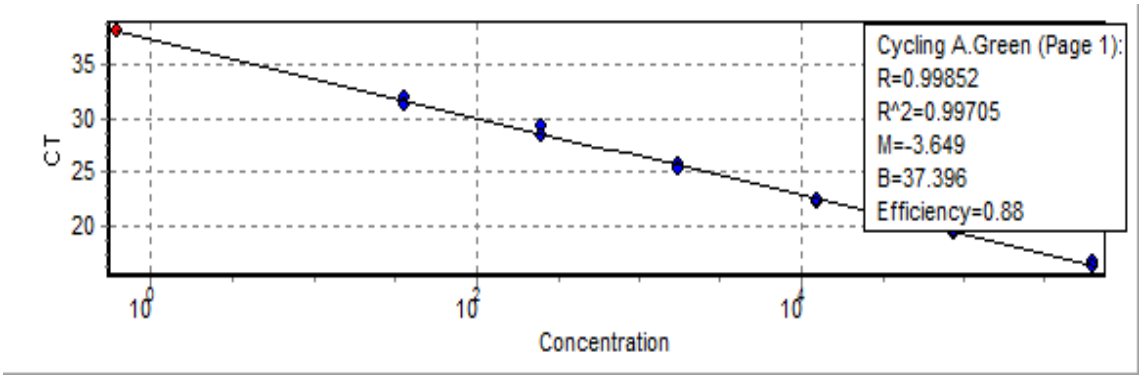
Amplicon-specific standard curves were generated for each gene of interest. Six serial dilutions of linearized plasmids diluted 1 in 7 was set up for each gene. An optimized standard curve should meet the following criteria: r^2 of >0.98, amplification efficiency of as close to 100%, and a single peak in the melt curve to confirm specificity. The efficiency of the PCR is a measure of the rate at which the polymerase converts the reagents in the reaction to amplicon; ideally at a rate at which the number of copies double after each cycle (Bustin, 2004; Bustin *et al.*, 2009). The r^2 (coefficient of determination) is derived from the equation of the linear regression line constructed from the starting quantity of the template against the Cq values (Taylor *et al.*, 2010). It indicates how good the line fits the data. The specificity of the reaction was represented by a single sharp peak in the melt curve. This was confirmed further by running the samples on agarose gel to reveal that the product was the expected size.

All the parameters required to ensure the reliability of real time RT-qPCR assay specified in the MIQE guidelines were completed. The efficiency of the reaction for all genes was at least 90%, the r^2 was >0.98, and melt curves yielded single peaks. These features are exemplified in Figure 3.9 and 3.10 showing the difference between a non-optimized and optimized standard curve. Details on the optimized conditions for each gene are presented in Table 3.3.

A. Standard curve plot of fluorescence versus cycle



B. Quantification analysis. Concentration versus threshold cycle (CT) [11]



C. Melt curve analysis

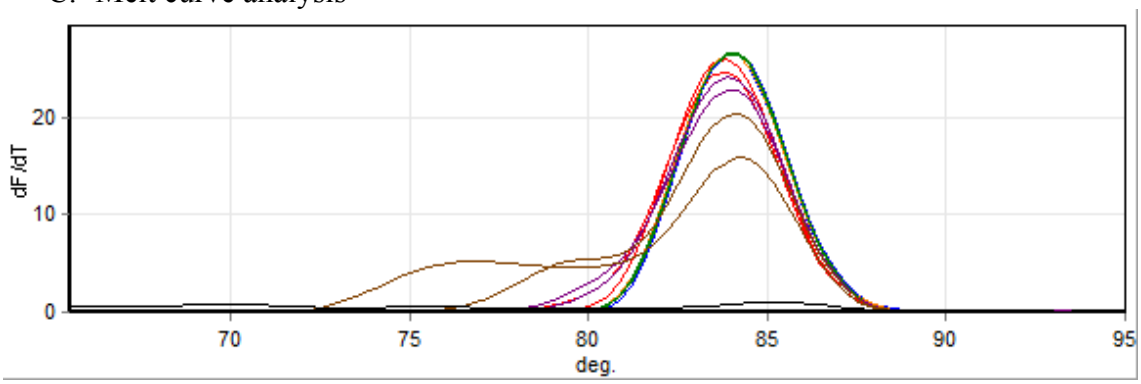
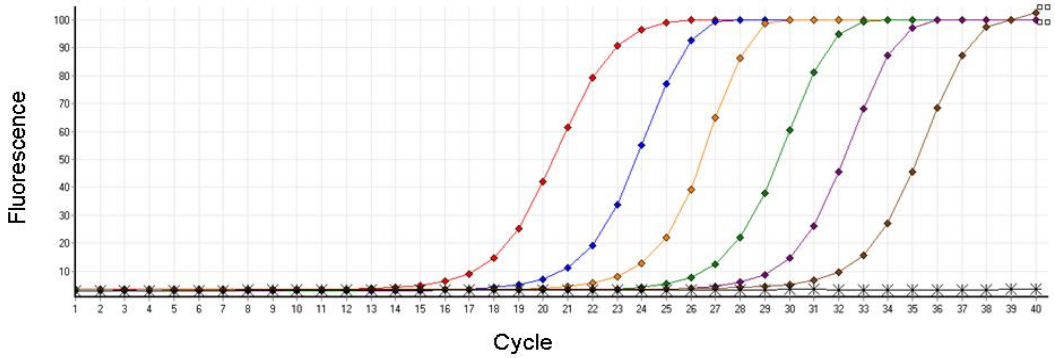


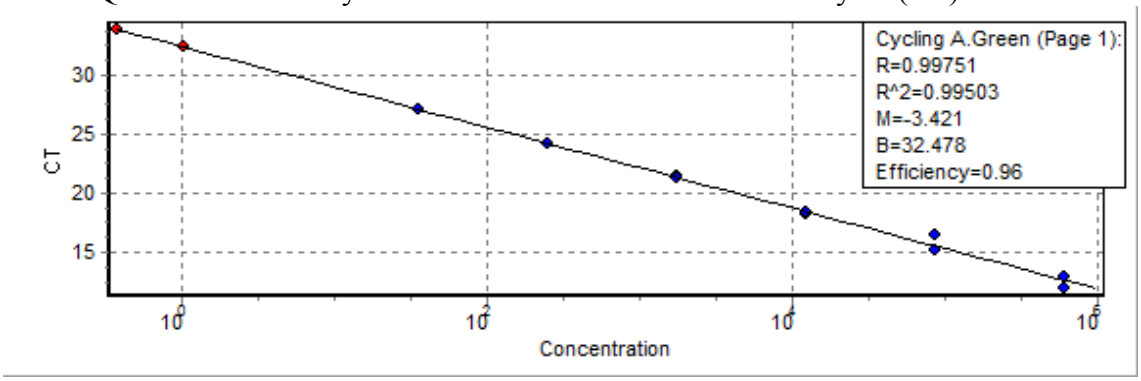
Figure 3.9 Construction of a non-optimized plasmid DNA standard curve. Amplification plot of standard curves (A) with six serial dilutions of linearized pDNA (shown in dotted colour lines) and a no-template control (represented by gray line with asterisk). The uneven gaps between serial dilutions contribute to the low efficiency in the standard curve (B). Single peak in the melt curve (C) indicates a single product but the 'shoulder' suggests non-specific amplification at higher dilution.

[11] Now appropriately termed quantification cycle (Cq) based on Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) guidelines

A. Standard curve plot of fluorescence versus cycle



B. Quantification analysis. Concentration versus threshold cycle (CT)



C. Melt curve analysis

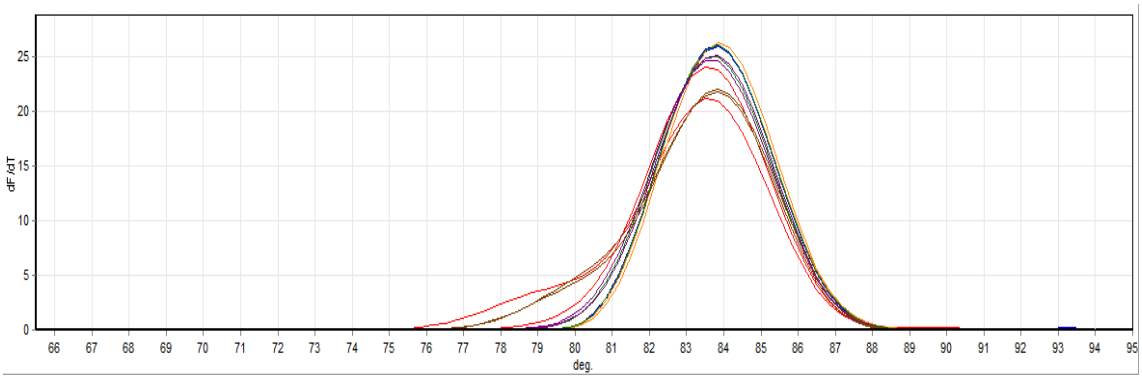


Figure 3.10 Construction of an optimized plasmid DNA standard curve. Optimization was performed on the same gene presented in Figure 1 with six serial dilutions of pDNA (shown in dotted colour lines) and a no-template control (represented by black line with asterisk). Amplification plot of standard curves (A) of the same gene in Figure 1 shows the earlier cycle at which the first standard achieved fluorescence signal. The efficiency is better at 96.0 % in the standard curve (B). The background noise caused by non-specific amplification was reduced as shown in the melt curve (C).

Table 3.3. Summary of optimized RT-qPCR assay reaction components and cycling conditions.

Gene	Primer conc (nm) For/Rev	$MgCl_2$ conc (mM)	Anneal temp (°C)	Reaction efficiency	Range of Cq values	R2
IL-2	600	2.0	66	1.02	10.68-24.36	0.99
IL-4	600	2.0	62	1.09	09.20-22.39	0.99
IL-6	500	4.0	62	0.92	12.45-27.38	0.99
IL-7R	600	2.0	63	0.99	11.61-25.90	0.99
IL-10	500	2.0	63	0.96	17.15-31.60	0.96
IL-12p40	500	2.0	63	0.93	13.75-28.37	0.99
IL-17A	600	2.0	62	0.96	15.13-29.56	0.99
IL-21	600	2.0	62	0.94	16.61-31.89	0.99
IL-23A	700	3.0	62	0.98	12.14-26.74	0.99
IL-25	450	2.5	62	0.99	10.74-24.73	0.99
EBI3	1000	2.0	64	1.01	19.54-33.91	0.99
FOXP3	500	2.0	63	0.93	11.32-26.37	0.99
IFNγ	250	2.0	62	0.98	16.63-30.64	0.99
TGFβ	500	2.0	63	0.99	10.23-24.38	0.99
SDHA	300	2.0	62	0.90	10.25-33.09	0.99
YWHAZ	600	3.0	62	0.95	9.98-31.58	0.99

3.9 Conclusion

I have developed an optimized RT-qPCR to quantify the expression of a panel of cytokines and markers in the lymph nodes and abomasum of sheep. The assay was used to characterize the immune response to *Teladorsagia circumcincta* infection of lambs which are known to be resistant or susceptible to the worm.

Ovine sequences of the genes targeted for the assay were generated and verified. All optimization steps conforming to the MIQE guidelines were undertaken to ensure the reliability of the real time RT-qPCR assay.

The workable assay for the different genes has primer and magnesium concentration that ranged from 250-1000 nm and 2–4 mM respectively, and annealing temperature of 62ºC–66ºC. The cDNA template that will be used for the assay was optimized at a ratio that range from 1:10 to 1:40. At these conditions, all RT-qPCR reactions have an efficiency of >90% and r^2 of >0.98.

Chapter 4 Gene expression analysis and histopathology of resistant and susceptible lambs

4.1 Introduction

Genetic resistance to GI worms seems to operate through the development of an acquired immune response (Stear *et al.*, 1999b), chiefly with the production of IgA and IgE antibodies (Huntley *et al.*, 1998; Miller, 1996; Stear *et al.*, 2009; Stear *et al.*, 1996). Consequently, it takes both parasite exposure and time before resistance to nematode challenge develops. The original phenotypic study (Beraldi *et al.*, 2008), from which the current project developed, has shown that trickle infection with *T. circumcincta* led to a spectrum of response based on abomasal worm numbers, FEC and IgA antibody levels. Low FEC and adult worm numbers were significantly associated with high IgA antibody levels (Beraldi *et al.*, 2008), suggesting that IgA is responsible for host control of helminth infection by inhibition of worm development and reduction in egg production (Gill *et al.*, 1993b; Kanobama *et al.*, 2002; Smith, 2007a; Stear *et al.*, 1999). It also prevents larval establishment and therefore reduces tissue injury from helminth-induced inflammation (Bisset *et al.*, 1996; Pernthaner *et al.*, 2005b; Pettit *et al.*, 2005; Stear *et al.*, 1995; Strain *et al.*, 2002).

The critical role of T cells in helminth immunity has been well documented. Protection with gastrointestinal parasites is a consequence of the differentiation of $CD4^+$ T-helper lymphocytes into TH2 cells (Balic *et al.*, 2000b; Lacroux *et al.*, 2006). It is supposed that the effector mechanisms in lambs that clear *T. circumcincta* infection are due largely to IL-4, IL-5, and IL-13. These cytokines have been shown to promote B cell proliferation and IgE and IgA production (Kooyman *et al.*, 2000; Stear *et al.*, 2004). However, there are ambiguities in the distinct segregation of TH cell subsets associated with resistance and susceptibility in many host and nematode species. Modified TH2/TH1 response, Treg activation and its opposing balance with

TH17 have been linked with host response to parasites (Bettelli *et al.*, 2006; Craig *et al.*, 2007; MacKinnon *et al.*, 2009; Wen *et al.*, 2011). It is highly probable that T cells have a major influence on how the host effectively eliminates worm infection as seen in mice and humans. In selected sheep lines, nematode resistance was abrogated following treatment with anti-CD4 monoclonal antibody (Gill *et al.*, 1993a; Pena *et al.*, 2006).

It was also hypothesized that the immunological response in the development of resistance and susceptibility to *T. circumcincta* is associated with the different TH cell subsets. With the same group of animals used for histopathology, 14 cytokines and genes related with the differential activation of TH1, TH2, TH17, and Treg subsets were evaluated for their distinctive expression in the most resistant and most susceptible lambs. Significant differences in expression of any of the 14 genes in the abomasal lymph node and mucosa shall formed the basis in selecting genes for further study.

4.2 Histopathology of abomasum

Representative samples of abomasal tissues from 15 lambs used in relative quantification analysis were characterized for histopathologic changes. These consisted of five each of uninfected controls and infected resistant and susceptible lambs. Improperly fixed tissues of some samples were excluded as they showed some degrees of deterioration that would render them unsuitable for histopathological examination. Enumeration of mucosal mast cells was not performed because of very weak or non-specific staining observed in tissues stained with toluidine blue. Uninfected controls did not show any remarkable lesion; the mucosal epithelium was generally intact and the structures were apparently normal (Figure 4.1). However, very mild inflammation characterised by small foci of lymphocytic infiltrates in the lamina propria and the basal layer were noted in sections from one lamb. In resistant animals, minor pathological changes were noted with low level lymphocytic infiltrate and few eosinophils and neutrophils associated with the gastric glands (Figure 4.2); pre-adult stages of the nematode were not detected within the glands. Oedema was also observed in some areas with vacuolation in the mucosal layer surrounded with few lymphocytes, neutrophils, eosinophils, and cellular debris suggestive of larval migration (Figure 4.3). In the abomasal mucosa of susceptible lambs, major pathological changes were associated with extensive infiltration primarily of lymphocytes, and few eosinophils and neutrophils. These aggregates of cells covered larger areas of the mucosa and even extended into the submucosal layer (Figure 4.4 A and B). Few plasma cells were also found in these sites.

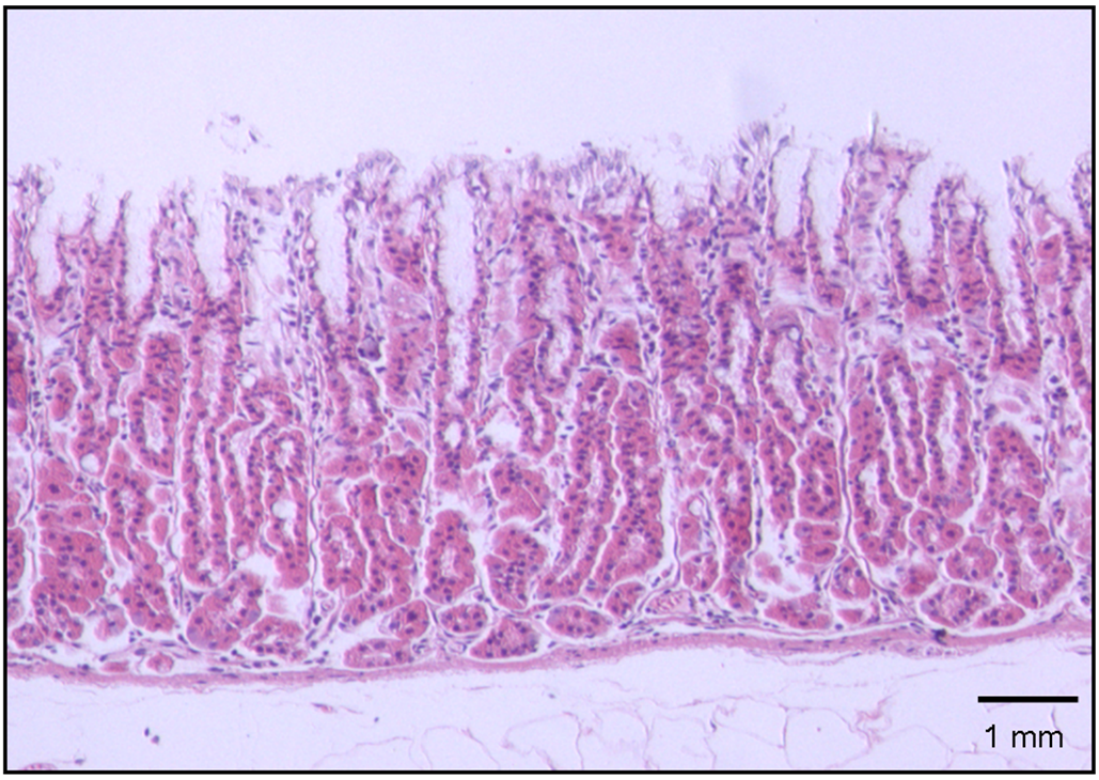


Figure 4.1 H&E section of the abomasum from uninfected control sheep showing normal mucosal epithelium (100X)

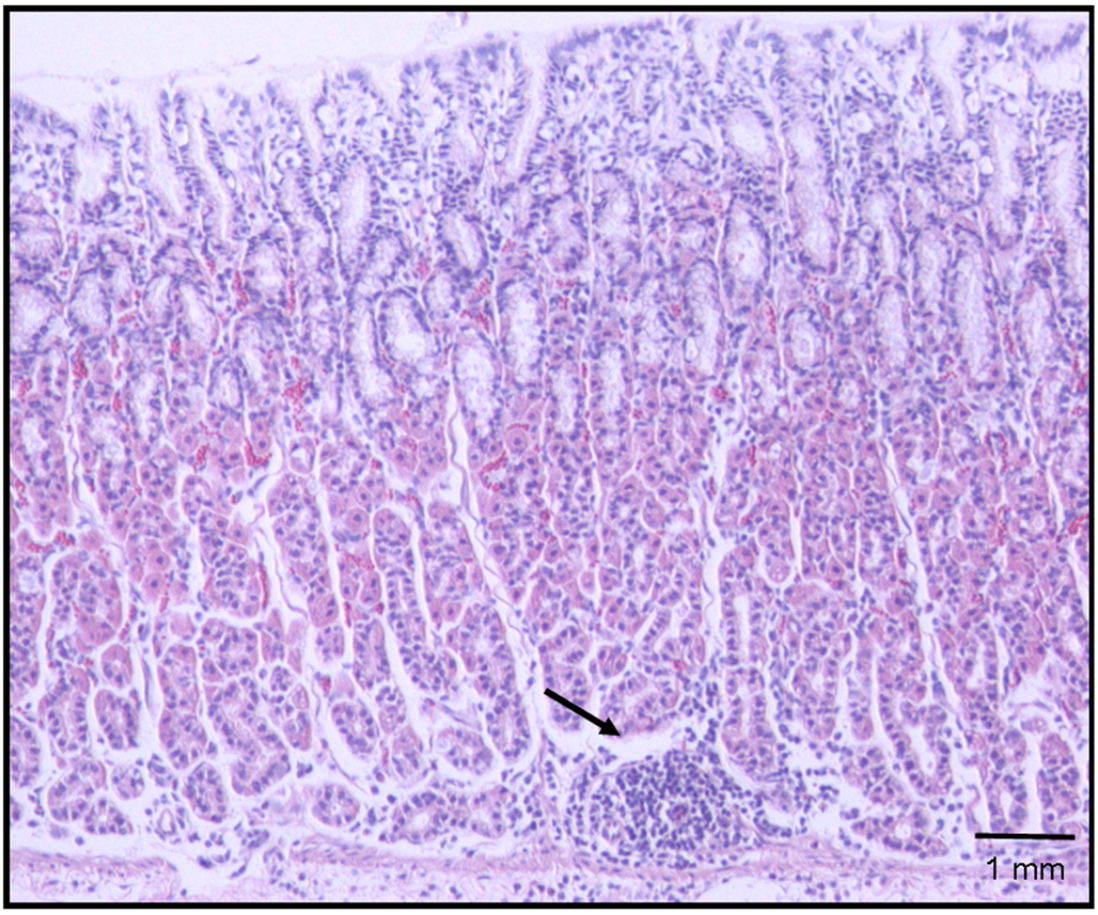


Figure 4.2 H&E section of the abomasum from infected resistant sheep showing focal area of lymphocytic infiltration (arrow) (100X).

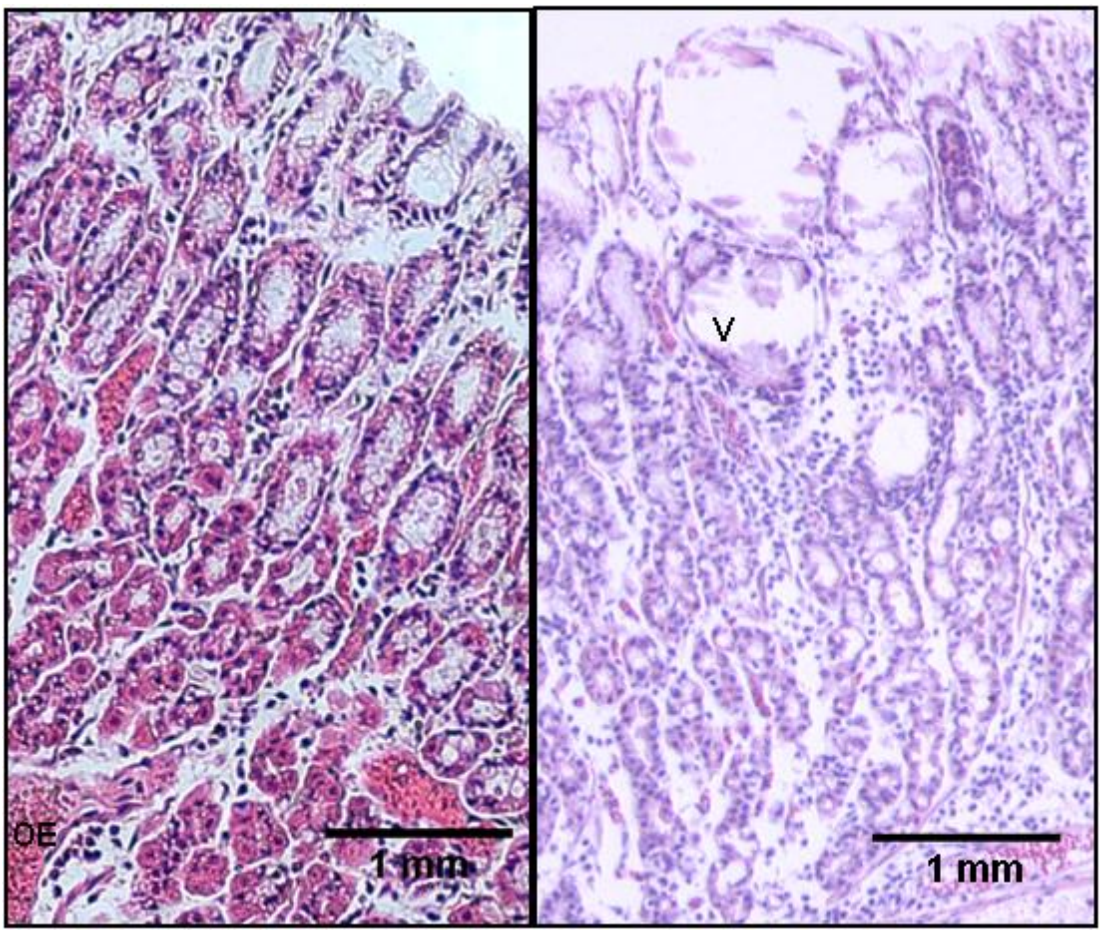


Figure 4.3 H&E sections of the abomasum from susceptible sheep showing mucosal (M) and submucosal (SM) infiltration of inflammatory cells primarily lymphocytes (L) (100X).

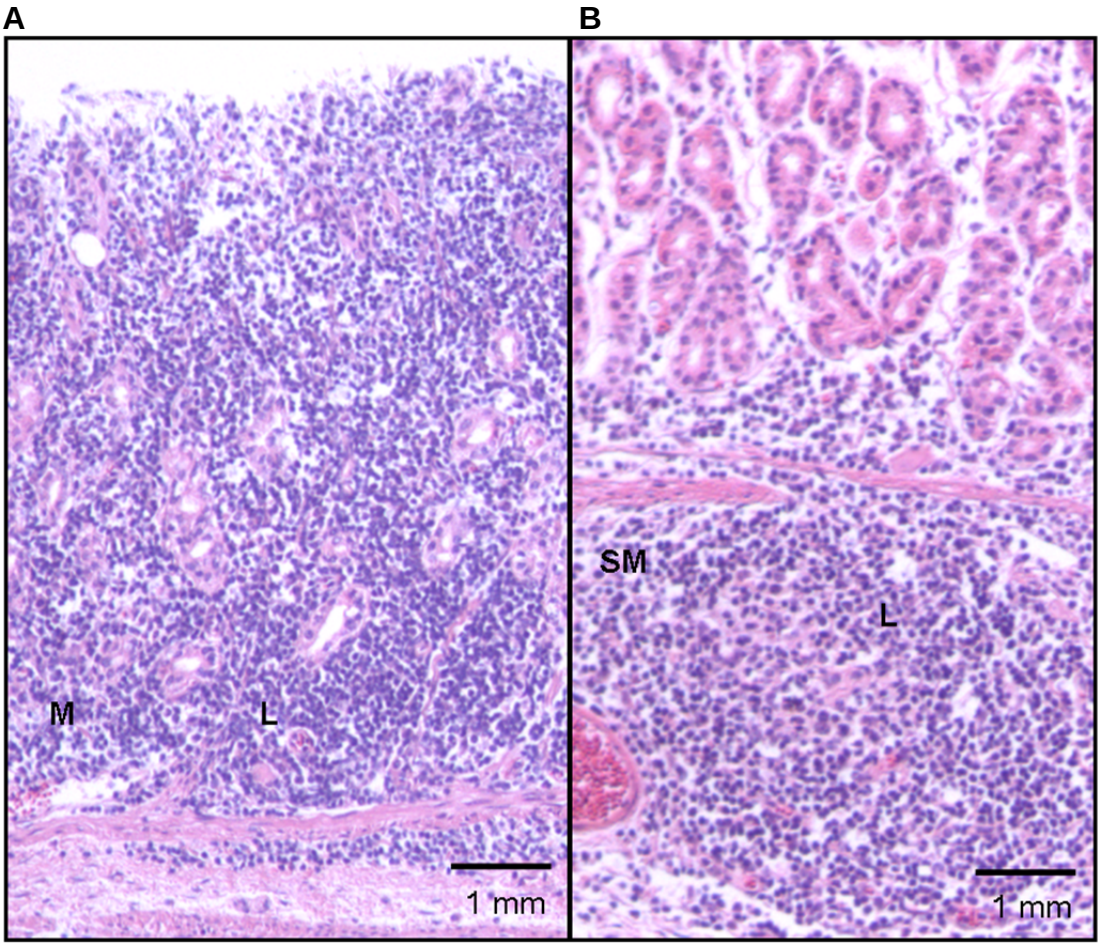


Figure 4.4 H&E section of the abomasum from infected susceptible lambs showing oedema (OE) in mucosal layer (A). Vacuolations (V) were evident but were empty of pre-adult stages (B). These are surrounded with lymphocytes, neutrophils, eosinophils, and cellular debris (100X).

4.3 Relative transcript expression in resistant and susceptible sheep

4.3.1 Fold change in abomasal lymph node

Real time RT-qPCR analysis was performed in resistant and susceptible lambs on 14 genes. Fold changes between groups and the P-values are illustrated in Figure 4.7 and summarized in Table 4.1.

Statistical analysis was performed on fold changes between three groups only on 11 genes. IL4, IL25 and IL17A were excluded from the analysis because their quantification cycle (Cq) values were outside the standard curve (Figure 4.5). This indicates that their transcript levels were outside the dynamic range of linearity of the assay and their expression is too low to be accurately quantified. Expression levels should at least be within the quantifiable range as exemplified in Figure 4.6. Significant differential expression between groups was obtained for IL6, IL21, EBI3, FOXP3 and TGFB1. No significant differences were found with any comparisons for IL2, IL7R, IL10, IL12B, IL23A, IL25 and TGFB1.

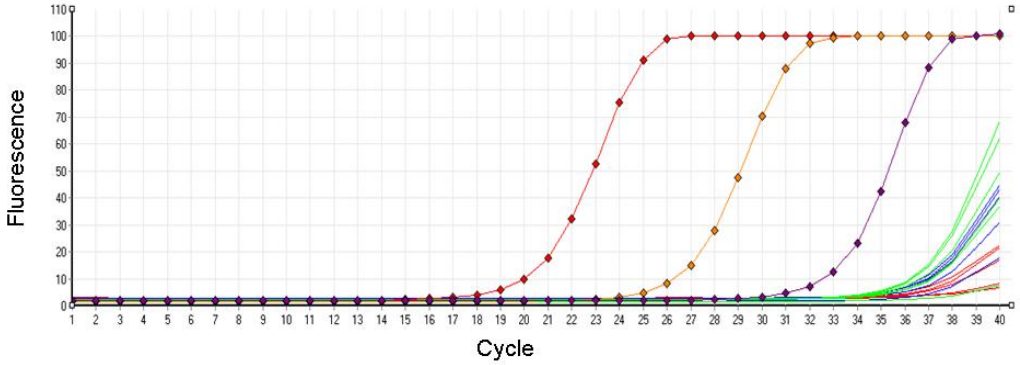
There was significant up-regulation of IL6 and IL21 in susceptible vs resistant as illustrated in Figure 4.7. The expression of both genes was higher in susceptible vs resistant with fold changes of 1.95 for IL6 ($P = 0.01$) and 2.24 for IL21 ($P = 0.03$). Similar trend was observed in susceptible vs control where susceptible had significantly higher IL6 and IL21 expression with fold changes of 2.30 ($P < 0.01$) and 2.06 ($P = 0.03$) respectively. Resistant vs control showed no significant differences for IL6 and IL21. Fold changes in resistant vs control for IL6 and IL21 were 1.25 ($P = 0.96$) and -1.01 ($P = 0.99$) respectively.

Table 4.1 Relative quantification of mRNA transcripts in abomasal lymph node

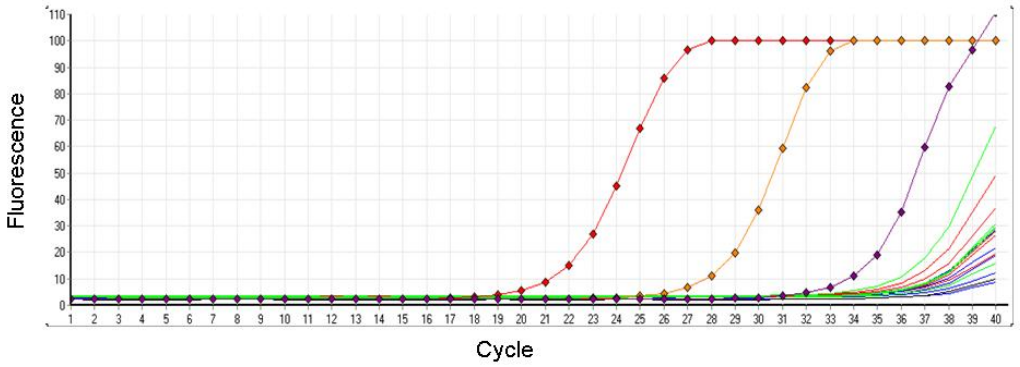
	Susceptible vs Control		Resistant vs Control		Susceptible vs Resistant	
Gene	fold change*	P-value	fold change	P-value	fold change	P-value
IL-2	1.58 ± 0.51	0.20	1.31 ± 0.66	0.60	1.32 ± 0.43	0.68
IL-4	ND		ND		ND	
IL-6	**2.30 ± 0.43**	**<0.01**	1.25 ± 0.45	0.88	**1.95 ± 0.36**	**0.01**
IL-7R	1.04 ± 0.28	0.10	-1.01 ± 0.54	0.96	1.17 ± 0.31	0.98
IL-10	1.73 ± 0.60	0.16	1.51 ± 0.29	0.42	1.16 ± 0.40	0.78
IL-17A	ND		ND		ND	
IL-12B	-1.23 ± 0.25	0.74	1.15 ± 0.69	0.90	-1.25 ± 0.24	0.48
IL-21	**2.06 ± 0.78**	**0.03**	-1.01 ± 0.48	0.99	**2.24 ± 0.85**	**0.03**
IL-23A	1.40 ± 0.19	0.23	1.19 ± 0.36	0.76	1.22 ± 0.16	0.58
IL-25	ND		ND		ND	
EBI3	**-2.56 ± 0.19**	**0.02**	-1.19 ± 0.46	0.63	-1.85 ± 0.27	0.10
FOXP3	**1.68 ± 0.33**	**0.04**	**1.91 ± 0.43**	**<0.01**	-1.11 ± 0.18	0.59
IFNγ	**-1.66 ± 0.23**	**0.04**	-1.22 ± 0.22	0.40	-1.32 ± 0.29	0.38
TGFβ	1.51 ± 0.28	0.22	1.46 ± 0.49	0.16	1.08 ± 0.20	0.98

* Fold change is the ratio of normalized mean expression between groups. Means of the non-infected controls were used as the calibrator and were arbitrarily assigned a value of 1.0 (using the $2^{-\Delta\Delta}$Cq method) and compared with the infected animals. ND - not detected; IL-4, IL-17A and IL-25 levels were outside the dynamic range of linearity of the assay and were too low to be accurately quantified and therefore valid comparisons could not be made. Bold is $P < 0.05$.

A. IL4



B. IL25



C. IL17A

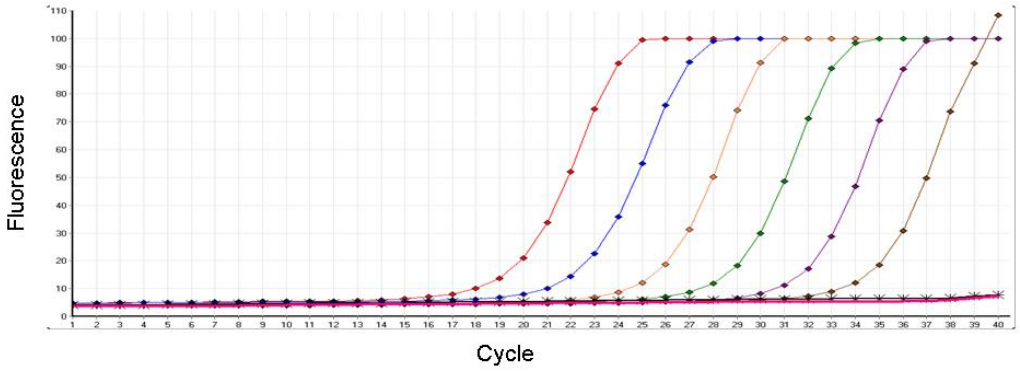
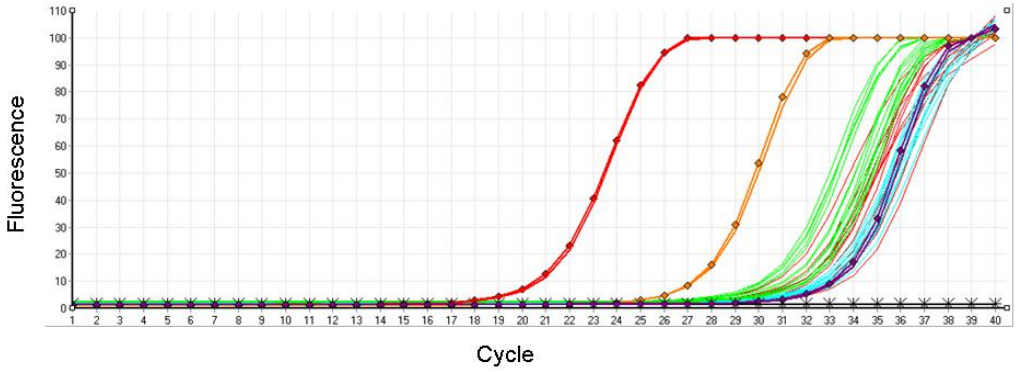


Figure 4.5 Amplification plots of lowly-expressed IL4 and IL25 (A &B) in 15 lymph node samples and IL17A (C) in pooled samples of susceptible lambs (replicate view). Lines with dots are pDNA standards and undotted lines represent control (red), resistant (blue) and susceptible (green) lambs with Cq values >35 indicating levels outside the dynamic range of quantifiable expression. No-template controls are represented by lines with asterisks (*).

A. IL21 expression in abomasal lymph node



B. TGFβ expression in abomasal mucosa

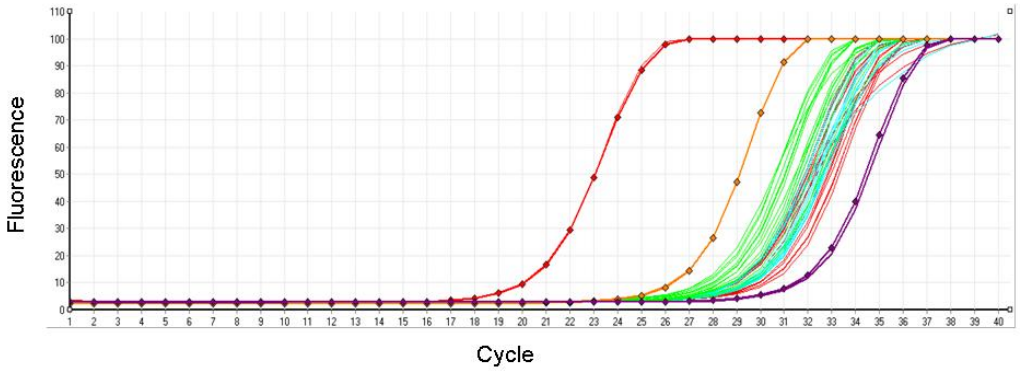


Figure 4.6 Amplification plots of genes within the quantifiable range with relative expression RT-qPCR. 15 lymph node samples representing control (red), resistant (blue) and susceptible (green) lambs are shown in thin lines. pDNA standards are represented by three thick lines. Cq values are < 35 indicating levels within the dynamic range of quantifiable expression for most of the samples.

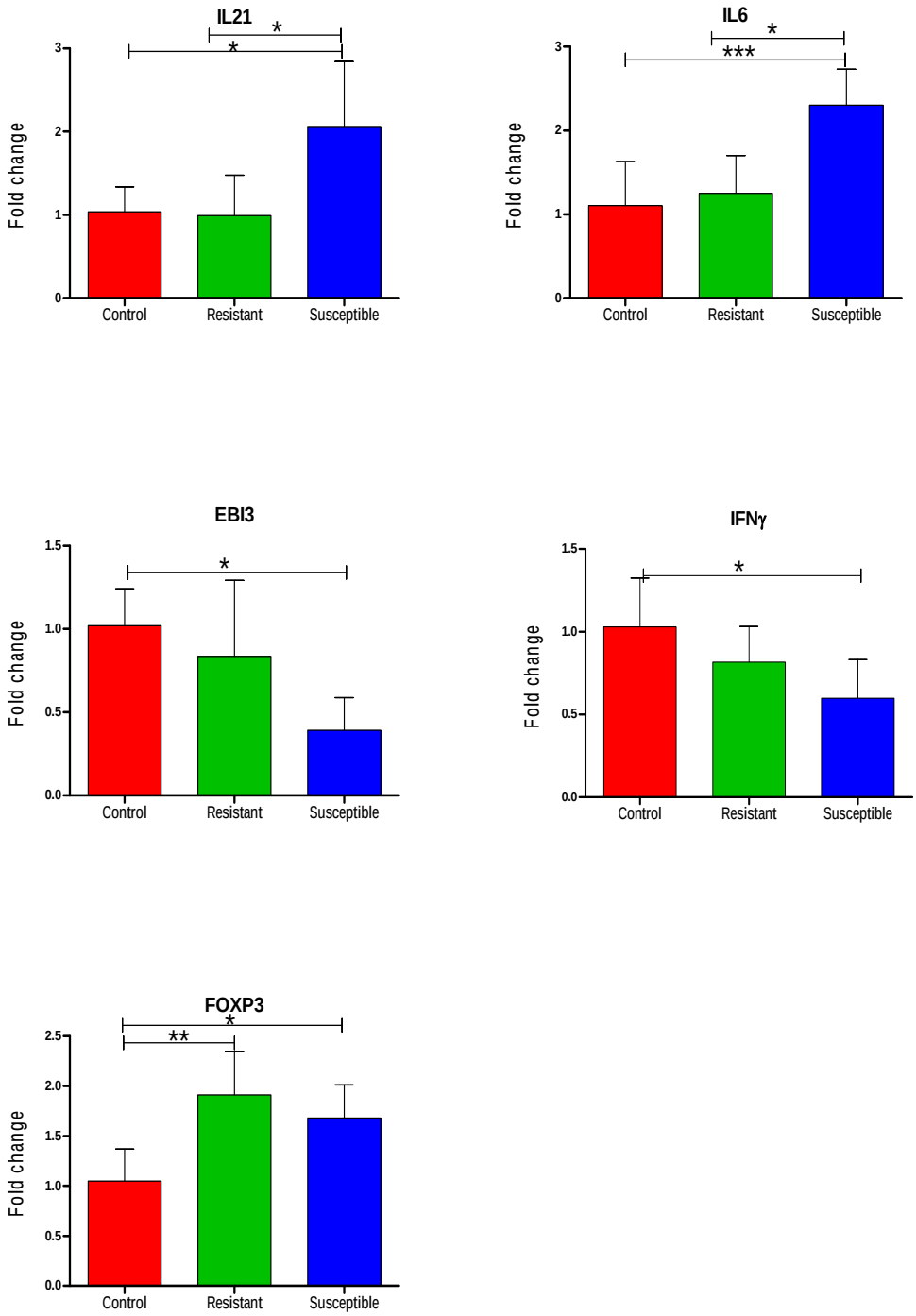


Figure 4.7 Relative expression of genes in the abomasal lymph node (ALN) of non-infected control (n=5) and infected resistant (n=5) and susceptible (n=5) sheep**.** Results are presented in fold change relative to the calibrator (mean of uninfected control) which were arbitrarily assigned a value of 1.0 (using the $2^{-\Delta\Delta}$Cq method). Brackets indicate statistical difference by one way ANOVA with Tukey Kramer's post hoc test at $P < 0.05$.

The fold change comparisons of EBI3, FOXP3 and IFNG between the three groups are displayed in Figure 4.7. EBI3 expression was lowest in susceptible lambs resulting in a significant -2.56 fold change in susceptible vs control comparison ($P = 0.02$). EBI3 levels in resistant vs control had fold changes of -1.19 and -1.85 in susceptible vs resistant which were both non-significant at $P > 0.10$.

There was a trend towards significant up-regulation in FOXP3 expression for both resistant and susceptible vs control. FOXP3 levels were elevated in susceptible (1.68 fold change; $P = 0.04$) and resistant (1.91 fold change, $P < 0.01$) vs control. There was no significant difference in FOXP3 expression in resistant vs susceptible with fold change of -1.11 ($P = 0.59$).

Statistically significant IFNG levels were found in susceptible vs control (fold change -1.66, $P = 0.04$). All other group comparisons for IFNG were not significant. Fold changes were measured at -1.22 ($P = 0.40$) in resistant vs control and -1.32 ($P = 0.38$) in susceptible vs control.

No significant differences were found across all group comparisons which include IL2, IL7R, IL10, IL12B, IL-23A, and TGFB1. IL2 had expression levels at1.3-fold change difference in resistant vs control ($P = 0.60$) and susceptible vs resistant ($P = 0.68$); susceptible lambs had a fold change of 1.58 ($P = 0.20$) vs control. There was only a slight difference in IL-7R expression with susceptible having fold changes of 1.04 ($P = 0.10$) vs control and 1.17 ± ($P = 0.98$) vs resistant. Resistant lambs showed almost parallel levels of IL7R compared to control group with fold change of -1.01 ($P = 0.96$).

There was consistent non-significant changes ($P<0.05$) in the expression of IL10, IL23A, and TGFB1 across all comparisons. IL10 levels had fold changes of 1.73 ($P = 0.60$) in susceptible vs control, and 1.16 ($P = 0.78$) vs resistant. A 1.51-fold difference was noted in resistant vs control but were not statistically significant ($P = 0.42$). Fold changes of IL23A were 1.40 ($P = 0.23$) in susceptible vs control, 1.19 ($P = 0.76$) in resistant vs control, and 1.22 ($P = 0.58$) in susceptible vs resistant.

Analysis on the expression levels of TGFB1 yielded fold changes of 1.51 ($P = 0.22$) in susceptible vs control and 1.46 ($P = 0.16$) in resistant vs control. TGFB1 transcripts in susceptible vs resistant had fold change of 1.08 ($P = 0.98$). IL12B in susceptible had fold change of about -1.20 vs control and resistant; no significant difference was noted in these comparisons at $P > 0.25$. On the other hand, resistant lambs had a fold change of 1.15 vs control which was also not statistically significant ($P = 0.48$).

4.3.2 Fold change in abomasal mucosa

Only six genes were included in the expression analysis of the abomasal mucosa since eight others were outside the level of detection as defined in 4.2.1. Table 4.2 shows the differential expression of IL6, IL7R, IL10, EBI3, IFNG, and TGFB1 in the abomasum of uninfected control and infected resistant and susceptible groups. Statistically significant differences between groups were noted for IL6 and TGFβ1 (Figure 4.9). No significant differences were found with any comparison for IL7R, IL10, EBI3, and IFNG.

Table 4.2 Relative quantification of mRNA transcripts in abomasal mucosa

Gene	Susceptible vs Control		Resistant vs Control		Susceptible vs Resistant	
	fold change ± sd	P- value	fold change ± sd	P- value	fold change ± sd	P- value
IL6	**4.44 ± 1.91**	**<0.01**	2.48 ± 0.81	0.23	1.79 ± 2.36	0.07
IL7R	-1.33 ± 0.19	0.33	1.00 ± 0.41	0.74	-1.33 ± 0.45	0.74
IL10	1.00 ± 0.30	0.66	-1.54 ± 0.18	0.19	1.53 ± 1.63	0.60
EBI3	-1.43 ± 0.40	0.33	-1.16 ± 0.35	0.70	-1.22 ± 1.12	0.78
IFNγ	2.03 ± 1.30	0.89	1.44 ± 0.76	0.94	1.41 ± 0.70	0.70
TGFβ1	**2.53 ± 0.35**	**<0.01**	1.35 ± 0.32	0.41	**1.88 ± 0.12**	**<0.01**

***** Fold change is the ratio of normalized mean expression between groups. Means of the non-infected controls were used as the calibrator and were arbitrarily assigned a value of 1.0 (using the $2^{-\Delta\Delta}$Cq method) and compared with the infected animals. Bold is $P < 0.05$.

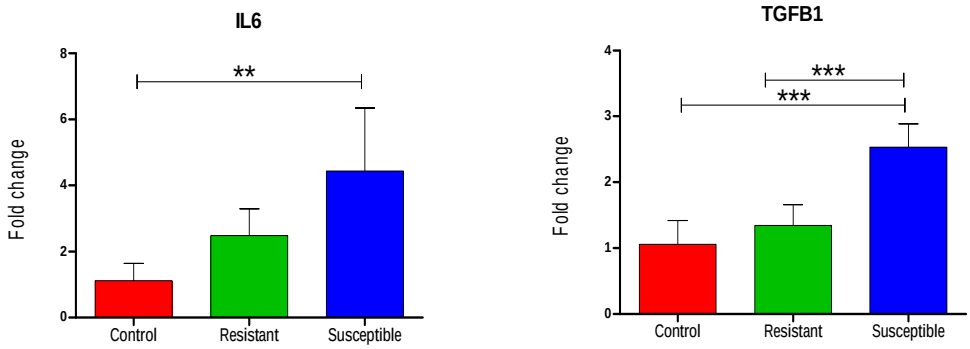


Figure 4.8 Relative expression of genes in the abomasal mucosa (ABM) of non-infected control (n=5) and infected resistant (n=5) and susceptible (n=5) sheep**.** Results are presented in fold change relative to the calibrator (mean of uninfected control) which were arbitrarily assigned a value of 1.0 (using the $2^{-\Delta\Delta Cq}$ method). Brackets indicate statistical difference by one way ANOVA with Tukey Kramer's post hoc test at $P < 0.05$.

IL6 was highest in susceptible with significant ($P < 0.01$) 4.44 fold change vs control. Susceptible had a fold change of 1.79 vs resistant which was marginally non-significant ($P = 0.07$). A statistically non-significant ($P = 0.23$) difference in fold change was also observed in IL6 expression in resistant vs control with a fold change of 2.48. It must be noted that some Cq values of IL6 for 15 samples in the abomasal mucosa were in the borderline of standard curve plots but were still included in the analysis as most samples were quantifiable. Consequently, variations were observed as reflected in the high standard deviation of the fold change between susceptible and resistant.

Significant up-regulation in TGFB1 expression was observed in susceptible lambs resulting in fold changes of 2.53 ($P <0.01$) vs control and 1.88 ($P <0.01$) vs resistant. There was no significant difference ($P = 0.41$) between resistant and control with 1.35 fold change.

Levels of EBI3 expression in all between-group comparisons were not statistically significant. Fold changes were -1.43 ($P = 0.33$) in susceptible vs control, -1.16 ($P = 0.70$) in resistant vs control and -1.22 ($P = 0.78$) in susceptible vs resistant. For IL7R, susceptible had similar fold change difference of -1.33 vs control and resistant which was non-significant at $P = 0.33$ and $P = 0.74$ respectively. In resistant vs control, the fold change was 1.00 ($P = 0.74$).

No significant differences were noted across all comparisons for both IL10 and IFNG. Fold change comparisons of IL10 expression between groups varied from 1.00 in susceptible vs control, -1.54 in resistant vs control and 1.53 in susceptible vs resistant. All these comparisons were non-significant at $P > 0.19$. Fold changes for

IFNG were 2.03 in susceptible vs control (P = 0.89), 1.41 in susceptible vs resistant (P = 0.70) and 1.44 in resistant vs control (P = 0.94).

4.4 Summary and conclusion

The section aimed to determine the histopathology and fold change difference among 15 uninfected and infected resistant and susceptible lambs. This formed the basis in identifying transcripts included to produce copy number measurements that were correlated with phenotype data in all 55 lambs.

Major histopathologic changes were associated with massive lymphocytic aggregates in the abomasal mucosa and submucosa of susceptible lambs. Low level lymphocytic infiltrates were found in resistant lambs, while uninfected lambs (control) had no apparent associated lesions.

The study has determined the relative expression of 14 cytokines and markers in the abomasal lymph node and abomasal mucosa in a cohort of lambs with genetic predilection for resistance and susceptibility to *T. circumcincta*.

There was a significant differential expression in the abomasal lymph node for IL6 and IL21 being two-fold higher in the susceptible lambs compared with resistant lambs. FOXP3 was significantly up-regulated by two-fold in infected lambs compared with uninfected controls, but not between resistant and susceptible. Other observed differences in expression involved EBI3 and IFNG which were both down-regulated by two-fold in susceptible lambs compared to uninfected controls. There were no significant differences in any comparisons for IL2, IL7R, IL10, IL12B,

IL23A, and TGFB1. IL4, IL17A and IL25 are too lowly expressed to be quantified accurately.

The expression of only six genes out of the targeted 14 mRNA transcripts in the abomasal mucosa was successfully determined as the rest were below the threshold of accurate detection. TGFB1 had significantly higher levels in susceptible lambs by two-fold relative to resistant. There was an upregulated expression of TGFB1 and IL6 in susceptible lambs compared to control with fold changes of 2.5 and 1.9 respectively.

Based on the relative quantification analysis by RT-qPCR in the abomasal lymph node and abomasal mucosa, IL6, IL21, EBI3, IL23A, and TGFB1 were selected for absolute RT-qPCR and correlation analysis with phenotype data. The differential expression of IL6, IL21 and EBI3 suggests a TH17 response so it was deemed necessary to also look at IL23A and TGFB1 copy number transcripts despite its non-significant difference between group comparisons.

Chapter 5 Quantitative gene expression and correlation with phenotype

5.1 Introduction

Analysis on the differential expression of 14 cytokines at extremes of the response spectrum suggests that susceptibility is correlated with the activation of pro-inflammatory TH17 cells. From these experiments IL6, IL21, IL23A, EBI3 and TGFB1 were selected for further expression analysis. IL6 and IL21 were chosen because their expression was significantly increased in the ALN of susceptible lambs in comparison to resistant lambs (Table 4.1). EBI3 (as IL35) is a known inhibitor of TH17 and was considered for further investigation as it was significantly reduced in the ALN in the susceptible lambs in comparison to uninfected controls. TGFB1 (as TGFβ) was significantly increased in the abomasal mucosa of susceptible lambs and plays a critical role in CD4+ T cell differentiation including the development of TH17 cells (with IL6) (Bettelli *et al.*, 2006). Although IL23A did not show significant changes in the fold-change analysis it was included because of its critical importance in the expansion and maintenance of TH17 cells (Boniface *et al.*, 2008; Tang *et al.*, 2012). In this section, the immunological response was investigated more closely by looking at the differential expression of five cytokines in the full cohort of 55 lambs.

The significant relationship between IgA antibody levels, FEC, adult worm count, worm length and fecundity in *T. circumcincta* infection has already been identified (Beraldi *et al.*, 2008; Smith *et al.*, 1983; Stear *et al.*, 1995). However, how these factors relate to the development of acquired protective immunity as reflected in differentially expressed genes has not been elucidated and was explored in this section.

The aim of the study was to investigate further any significant relationship between resistant and susceptible lambs in the five cytokines linked with TH17 response. Absolute quantification analysis was performed to measure transcript expression in the full cohort of resistant and susceptible lambs for five genes. It was hypothesised that the expression of IL6, IL21, EBI3, TGFB1 and IL23A levels are correlated with the individual physiological parameters. In order to ascertain the relationship, copy number measurements of 45 infected lambs were correlated with adult worm count, FEC, IgA levels and body weight. Differences in gene expression and its correlation with phenotype could provide insights into the immunological basis of resistance to *T. circumcincta.* It also offers a comprehensive view of the dynamics of T-cell immunity as it relates to immunology, pathology and physiology of a mature protective immune response.

5.2 Absolute quantification by Real-time RT-qPCR in ALN

5.2.1 Experimental set-up

All 55 animals were included in absolute quantification analysis (Table 5.1). These were analysed in four groups: 10 uninfected controls (zero FEC and adult worm count); a resistant group of 15 lambs with the lowest adult worm count and FEC (mean adult worm count = 59; mean FEC = 2); the 15 most susceptible lambs with the highest adult worm count and FEC (mean adult worm count = 5,167; mean FEC = 288); and 15 intermediate sheep with mean adult worm count = 1,508; mean FEC = 82 (Table 5.1).

All samples were run in triplicates for three sets of cDNA templates from independent reverse transcription reactions. With this set up, it was not possible to

perform analysis of the samples for each gene in a single run as the number of samples could not be accommodated in either a 72 or 100 well rotor disc. To ensure that inter-run differences will not influence the copy numbers generated from each run, samples for each gene were analysed on the same day using the same standard dilution plasmids for each gene. In addition, the same threshold levels were applied for each gene in generating raw copy numbers.

5.2.2 Copy numbers of mRNA transcripts in the ALN

Copy numbers for each transcript were calculated as described in Section 2.4.2. Table 5.2 shows the mRNA transcript expression in copy number measurements and the statistical analysis (Table 5.3) of the ALN in 55 lambs. Significant differences in transcript expression between any of the groups were observed only with IL6, IL21 and IL23A (Figure 5.1). TGFB1 and EBI3 did not show any differential expression among any group comparisons (Figures 5.1).

The copy number of IL6 transcripts in the resistant group was 10,550 ± 2,941 (± SD) per μg of total RNA, significantly lower than the susceptible 14,299 ± 4,447 (P = 0.02). IL6 levels in the intermediate group were 9,108 ± 2,983, significantly lower than both the uninfected controls with 13,525 ± 3,398 (P = 0.02) and the susceptible group (P = 0.02), but not significantly different to the resistant animals (P = 0.67).

Both IL21 and IL23A had a graded increase from the resistant to the susceptible group. IL21 levels in the uninfected controls were 5,159 ± 2,218 and in the resistant, intermediate and susceptible groups were 4,611 ± 1,407; 6,065 ± 2,715 and 7,894 ± 2,433 respectively. In all group comparisons for IL21, only the susceptible group showed significantly higher transcript levels compared to both the control (P = 0.02)

and resistant ($P < 0.01$) groups. IL23A levels in the controls were 522 ± 297, and in the resistant, intermediate and susceptible were 274 ± 115, 425 ± 283 and 773 ± 618 respectively; only the resistant and susceptible comparison was significantly different ($P < 0.01$).

There was no significant difference in any group comparison for EBI3 and TGFB1. Transcript levels of EBI3 varied from 9,282 ± 2,785 in the control group to 9,826 ± 4,405 in susceptible, 10,781 ± 4,405 in intermediate, and 9,377 ± 3,744 in resistant animals. There were comparable levels of TGFB1 in all four groups which had copy numbers of 255,521 ± 34,753 in the controls, 287,122 ± 59,198 in the resistant, 258,582 ± 112,920 in the intermediate, and 264,658 ± 28,484 in the susceptible lambs. There were no significant differences between the four groups for either EBI3 or TGFB1 at $P > 0.68$.

Table 5.1 Normalized copy numbers of cytokine transcripts in abomasal lymph nodes

Infection rank[a]	AWC	FEC (eggs/g)	IgA[c]	Body Wt (kg)	Copy numbers per μg total RNA				
					IL6	IL21	IL23A	EBI3	TGFB1
Uninfected Control mean adult worm count = 0, mean FEC = 0.									
0	0	0	0.027	35.5	14848	5812	1233	6217	277905
0	0	0	0.020	36	15740	1579	632	15224	273238
0[b]	0	0	0.177	36	11569	6316	372	10112	261563
0[b]	0	0	0.025	34.5	11545	3695	737	11085	255047
0	0	0	0.015	29	12031	3152	416	6638	264836
0[b]	0	0	0.288	27.5	19852	8863	560	11398	235090
0[b]	0	0	0.13	30	17499	4358	297	9455	219535
0[b]	0	0	0.031	36	12800	7373	202	7415	311237
0	0	0	0.146	33	8827	3762	416	8275	271048
0	0	0	0.077	32	10543	6682	350	7005	185708
Resistant mean adult worm count = 59, mean FEC = 1.67.									
1[b]	0	0	1.195	30	14505	5230	398	9278	285751
2	0	0	0.630	29	6236	3697	218	5605	319857
3[b]	0	0	0.798	36	15049	3593	271	10475	382993
4[b]	0	0	0.633	28	15805	7102	352	4134	380748
5[b]	0	0	0.077	30	9593	1847	415	12614	340326
6[b]	0	0	0.373	32	7643	4567	483	9750	285727
7	0	0	0.384	25	8151	3277	374	19023	242788
8	0	0	1.695	15	9121	3667	177	11453	210377
9	0	0	1.066	27	11025	7259	329	13117	210896

Table 5.1 cont'n

Infection rank[a]	AWC	FEC (eggs/g)	IgA[c]	Body Wt (kg)	Copy numbers per μg total RNA				
					IL6	IL21	IL23A	EBI3	TGFB1
10	80	0	0.856	30	7651	4549	272	6985	317280
11	100	0	0.126	29	7577	4494	224	7408	200657
12	100	0	0.547	32.5	11819	4186	134	7892	241734
13	100	25	0.154	34	12040	5403	81	7904	306587
14	200	0	0.782	27	10802	5658	232	9783	253920
15	300	0	0.706	29	11237	4642	151	5231	327194
I**ntermediate** mean adult worm count = 1508, mean FEC = 82.									
16	400	0	0.706	39	6150	4006	293	7901	247866
17	420	75	0.232	26	11377	9367	92	7268	267654
18	200	25	0.232	27.5	6463	7099	900	11941	172305
19	600	0	0.596	20	8101	5210	887	12495	185710
20	900	0	0.804	37	7826	4899	830	4309	172130
21	800	0	0.703	26	10711	5189	499	9157	198136
22	1200	0	0.142	35	10405	4737	405	16643	240921
23	1700	175	0.210	26	12801	13262	393	6642	235585
24	1200	0	0.161	28	14711	2135	746	10501	635008
25	2400	0	0.732	26	3336	5405	344	11582	190601
26	2300	175	1.510	34	11517	8153	174	3415	260633
27	2400	475	0.539	35	5971	7441	198	14135	223056
28	2400	75	0.183	31	9803	5942	131	11252	303635

Table 5.1 (cont'n)

Infection rank[a]	AWC	FEC (eggs/g)	IgA[c]	Body Wt (kg)	Copy numbers per μg total RNA				
					IL6	IL21	IL23A	EBI3	TGFB1
29	2600	100	0.259	36	7731	3591	199	16544	315039
30	3100	125	0.468	37.5	9719	4543	282	17926	230453
Susceptible	mean adult worm count = 5167, mean FEC = 288.								
31	3300	175	0.245	36.5	9208	11363	442	4387	251554
32	2900	225	0.219	30	19950	9988	2692	9936	316898
33	3800	100	0.073	35	16834	6704	1205	11897	270513
34	3900	250	0.840	35	13298	8004	1275	10973	273123
35	4200	275	0.060	28	12317	6188	1041	10722	296247
36	4700	150	0.033	34	12604	4217	800	11879	309652
37	5400	75	0.697	38	24497	8343	399	18275	227351
38	5300	250	0.151	27.5	16951	10784	522	10585	254183
39	4000	125	0.035	29	9193	5681	319	14154	240377
40	6000	200	0.126	34	9966	6403	498	16722	215693
41[b]	6000	200	0.451	36	10526	5708	247	5460	284781
42[b]	5200	525	0.155	37	17829	8497	467	5843	250540
43[b]	6200	200	0.047	27	17534	7250	658	5404	269353
44[b]	5300	950	0.209	22	11700	6381	661	6703	251621
45[b]	11300	625	0.141	37	12073	12900	371	4455	257977

[a]Lambs ranked according to adult worm count in the total abomasal contents at post-mortem.and FEC (eggs per gram wet faeces) at final collection

[b]Lambs selected for the fold-change analysis; [c] anti-*T. circumcincta* IgA concentrations calculated relative to standard curve

Table 5.2 Cytokine transcript expression in the gastric lymph node of *T. circumcincta* infected sheep (Copy number per μg total RNA)

	Control	Resistant	Intermediate	Susceptible
IL6	13525 ± 3398	10550 ± 2941	9108 ± 2983	14299 ± 4447
IL21	5159 ± 2218	4611 ± 1407	6065 ± 2715	7894 ± 2433
IL23A	522 ± 297	274 ± 115	425 ± 283	773 ± 618
EBI3	9282 ± 2785	9377 ± 3744	10781 ± 4405	9826 ± 4404
TGFB1	255521 ± 34753	287122 ± 59198	258582 ± 112920	264658 ± 28484

Table 5.3 Cytokine transcript expression in the gastric lymph node of *T. circumcincta* infected sheep (P-value of between-group comparisons)

	Control	Resistant	Intermediate
IL6			
Resistant	0.17		
Intermediate	**0.02**	0.67	
Susceptible	0.95	**0.02**	**<0.001**
IL21			
Resistant	0.93		
Intermediate	0.76	0.30	
Susceptible	**0.02**	**<0.01**	0.13
IL23A			
Resistant	0.39		
Intermediate	0.92	0.70	
Susceptible	0.38	**<0.01**	0.07
EBI3			
Resistant	1.00		
Intermediate	0.79	0.77	
Susceptible	0.99	0.99	0.91
TGFB1			
Resistant	0.69		
Intermediate	1.00	0.68	
Susceptible	0.99	0.82	0.99

Groupings were based on worm burden parameters as used in the experiment (Beraldi et al, 2008): control (n=10, mean adult worm count =0, mean FEC = 0), resistant (n=15, mean adult worm count =59, mean FEC = 1.67), intermediate (n=15, mean adult worm count =1508, mean FEC = 82), and susceptible (n=15), mean adult worm count =5167, mean FEC = 288). Bold is $P < 0.05$.

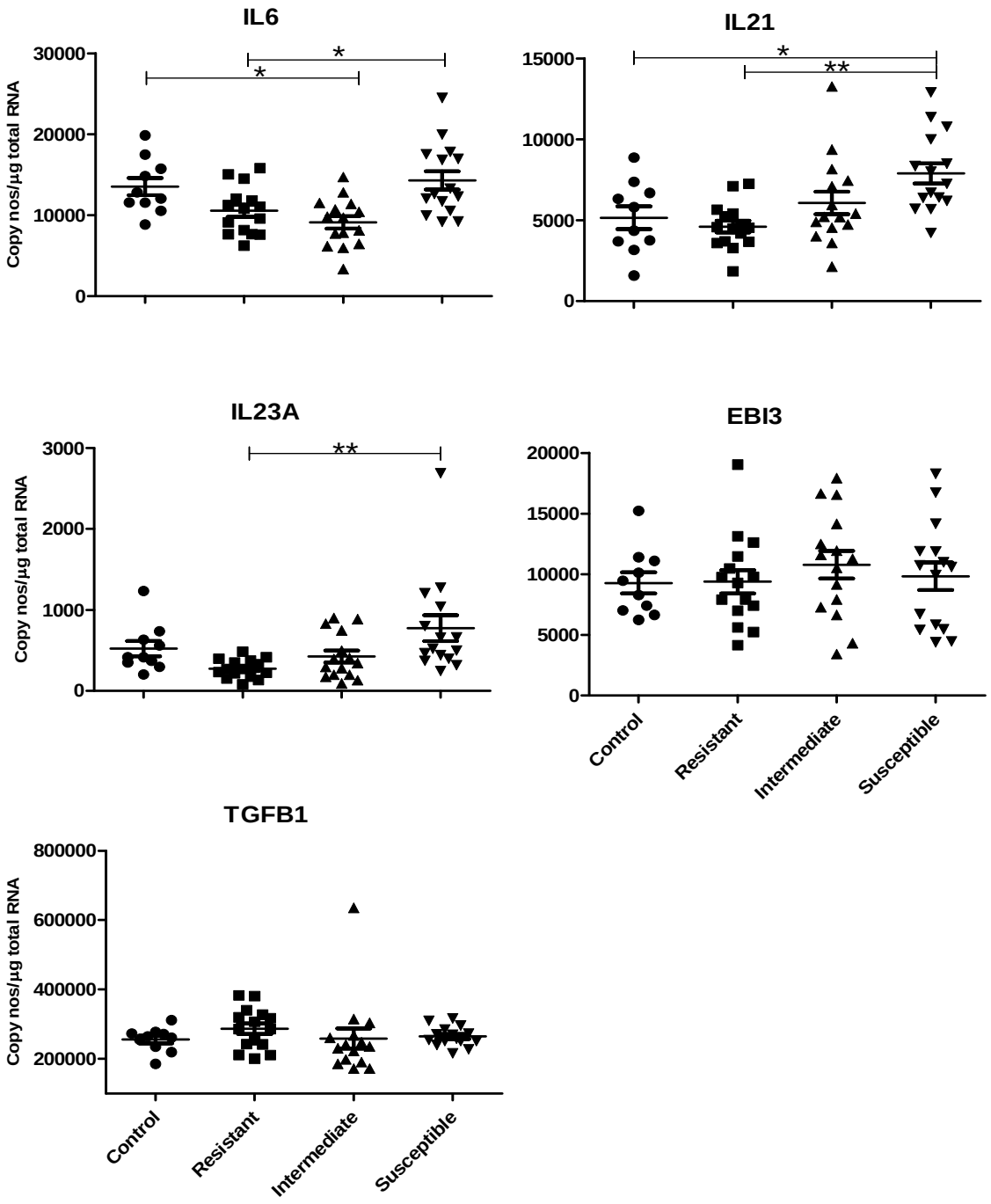


Figure 5.1 Copy numbers of gene transcripts in the abomasal lymph node of control, resistant, intermediate, and susceptible. Error bars are standard error of the mean (SEM) and brackets represent significant difference between group comparisons at $P < 0.05$ (*), $P < 0.01$ (**), and $P < 0.001$ (***). Significant differences between resistant and susceptible lambs in IL6, IL21 and IL23A expression are shown

5.3 Absolute quantification RT-qPCR in abomasal mucosa

IL6 and TGFB1 were chosen for copy number measurement in all 55 animals based on fold change comparisons of gene transcripts in the abomasal mucosa. Results are presented in Table 5.4.

5.3.1 Copy numbers of mRNA transcripts in abomasal mucosa

The expression level of TGFB1 transcripts in the susceptible group was 40,686 ± 11,964 and was significantly different ($P < 0.04$) to TGFB1 levels in the intermediate group (25,472 ± 7,910). Other TGFB1 and IL6 group comparisons did not show any significant differences (Figure 5.2). TGFB1 had copy numbers of 32,244 ± 19,250 in control and 31,125 ± 19,622 in resistant. IL6 showed low and highly variable copy numbers in control (1,516 ± 1,297), resistant (2,090 ± 3,841), intermediate (626 ± 327), and susceptible (2,928 ± 2,428).

Table 5.4 Cytokine transcript expression in the abomasal mucosa of *T. circumcincta* infected sheep

A. Copy number per μg total RNA

	Control	Resistant	Intermediate	Susceptible
IL6	1516 ± 1297	2090 ± 3841	626 ± 327	2928 ± 2428
TGFβ	32244 ± 19250	31125 ± 19622	25472 ± 7910	40686 ± 11964

B. *P* values of between-group comparisons

	Control	Resistant	Intermediate
IL6			
Resistant	0.94		
Intermediate	0.81	0.37	
Susceptible	0.50	0.78	0.06
TGFβ1			
Resistant	0.99		
Intermediate	0.69	0.74	
Susceptible	0.52	0.32	**0.04**

Groupings were based on worm burden as used in the experiment (Beraldi et al, 2008): control (n=10, mean adult worm count =0, mean FEC = 0), resistant (n=15, mean adult worm count =59, mean FEC = 1.67), intermediate (n=15, mean adult worm count =1508, mean FEC = 82), and susceptible (n=15), mean adult worm count =5167, mean FEC = 288). Bold is $P \leq 0.05$.

Table 5.5 Normalized copy numbers of cytokine transcripts in the abomasal mucosa

Infection rank[a]	IL6	TGFB1	Infection rank[a]	IL6	TGFB1
Uninfected control (mean AWC = 0, FEC = 0)			Infected intermediate (mean AWC = 1508, FEC = 82)		
0	30542	2005	16	18861	649
0	33438	451	17	40397	1095
0	85328	1081	18	36487	1399
0	18162	765	19	39243	1050
0	28409	1600	20	11513	220
0	28896	774	21	17602	372
0	22921	4532	22	30730	826
0	19716	2719	23	25778	694
0	27460	233	24	24269	658
0	27563	1003	25	24913	431
Infected resistant (mean AWC = 59, FEC = 1.67)			26	24832	331
1	35863	1389	27	18108	301
2	19601	15848	28	22095	399
3	8086	967	29	24601	430
4	39872	882	30	22645	530
5	16570	684	Infected susceptible (mean AWC = 5167, FEC = 288)		
6	18653	674	31	32431	539
7	15755	677	32	23906	473
8	16055	1474	33	42873	1384
9	21185	782	34	46871	4197
10	81533	1911	35	67306	2480
11	35172	1469	36	55097	3138
12	25147	680	37	36724	193
13	27684	742	38	41591	2905
14	61492	2362	39	26230	1349
15	44207	803	40	42629	7536
			41	42244	5309
			42	55352	4809
			43	37461	7360
			44	29454	1027
			45	30125	1226

Lambs ranked according to adult worm count (AWC) in the total abomasal contents at post-mortem and average faecal egg count (FEC) at the last week of data collection (Beraldi *et al.*, 2008).

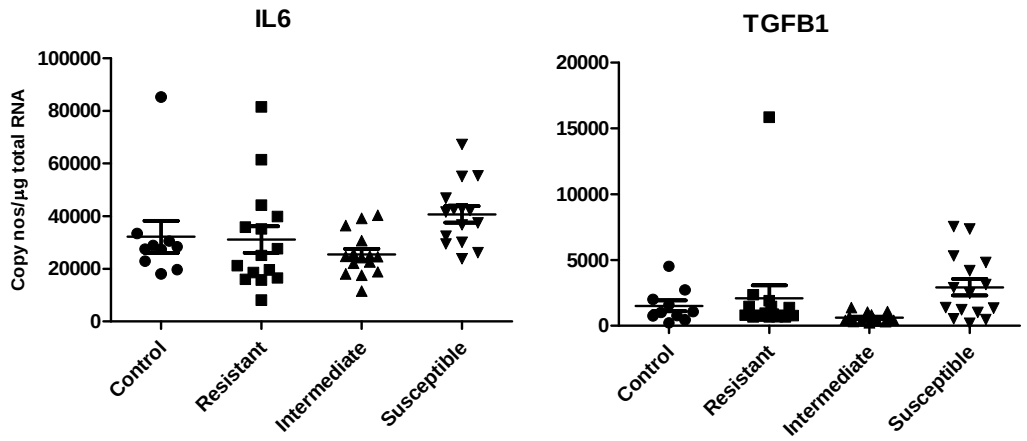


Figure 5.2. Copy numbers of gene transcripts in the abomasal mucosa of control (n=10, mean adult worm count =0, mean FEC = 0), resistant (n=15, mean adult worm count =59, mean FEC = 1.67), intermediate (n=15, mean adult worm count =1508, mean FEC = 82), and susceptible (n=15), mean adult worm count =5167, mean FEC = 288). Error bars represent the standard error of the mean (SEM). No significant differences between groups were found.

5.3.2 Correlation of transcript expression in the ALN and abomasal mucosa with physiological parameters

Fold change analysis revealed increased expression of IL6 and IL21 in the abomasal lymph node as well as IL6 and TGFB1 in the abomasal mucosa of the susceptible group of lambs in comparison to resistant animals (Section 4.4). It was proven in both fold change data and absolute copy number analysis that IL6 and IL21 are differentially expressed in the abomasal lymph nodes in resistant and susceptible lambs. The absolute quantification analysis has enabled the investigation of the relationship between cytokine transcript copy number and parameters used in assessing the phenotype characteristics of the lambs, which included FEC, adult worm count, worm-specific IgA levels, and body weight.

Using Spearman's rank analysis, significant correlations were observed (Table 5.6) in the quantitative expression of cytokine transcripts in the abomasal lymph node with FEC, adult worm count, and IgA. There was a positive correlation of IL6 (ρ = 0.408, P = 0.005), IL21 (ρ = 0.651, $P < 0.0001$), and IL23A (ρ =0.306, $P < 0.041$) transcript levels with FEC (Figure 5.3). Similarly, adult worm count was positively correlated with expression of IL6 (ρ = 0.348, P = 0.019), IL21 (ρ = 0.537, P = 0.0001) and IL23A (ρ = 0.378, P = 0.010) (Figure 5.4). In contrast, significant (P = 0.039) negative correlation (ρ = -0.308) was observed between IgA antibody levels and IL23A copy number (Figure 5.5). There were no significant correlations found in EBI3 and no significant correlations were established between IL6 ($P \geq 0.167$) and IL21 ($P \geq 0.167$) measurements with IgA and body weight respectively (Figures 5.7 and 5.8).

There was significant positive correlations found in TGFB1 transcript levels in the abomasal mucosa with adult worm count ($\rho = 0.425$, $P = 0.004$) and FEC ($\rho = 0.007$, $P = 0.034$). TGFB1 was negatively correlated with IgA ($\rho = 0.256$) but this was not statistically significant at $P = 0.09$ (Figure 5.6). No significant association was also noted in the expression of TGFB1 and body weight ($P = 0.97$). The correlation coefficient of IL6 ($\rho = -0.274$) with IgA suggests a negative correlation but this was marginally non-significant ($P = 0.07$); non-significant negative correlation was also noted between IL-6 and body weight ($\rho = -0.198$, $P = 0.193$) (Figure 5.7). No significant correlations were established for IL6 copy number and either adult worm count ($\rho = 0.142$) or FEC ($\rho = 0.156$) at $P \geq 0.305$).

Table 5.6 Correlation analysis of phenotypic parameters with cytokine transcript copy number in abomasal lymph node and abomasal mucosa

	Adult worm count		FEC		IgA		Body weight	
	ρ[a]	*P* value	ρ	*P* value	ρ	*P* value	ρ	*P* value
				Abomasal lymph node				
IL6	**0.348**	**0.019**	**0.408**	**0.005**	-0.210	0.167	0.066	0.664
IL21	**0.537**	**0.0001**	**0.651**	**<0.0001**	-0.135	0.377	0.031	0.838
IL23A	**0.378**	**0.010**	0.306	0.041	**-0.308**	**0.039**	-0.054	0.723
EBI3	0.003	0.982	-0.179	0.239	-0.164	0.283	-0.025	0.870
TGFB1	-0.128	0.402	-0.020	0.895	-0.125	0.414	0.138	0.365
				Abomasal mucosa				
IL6	0.142	0.353	0.156	0.305	-0.274	0.069	-0.198	0.193
TGFB1	**0.425**	**0.004**	**0.317**	**0.034**	-0.256	0.090	0.007	0.966

Bold; significant correlation ($P \leq 0.05$)

[a]ρ, Spearman's rank correlation coefficient

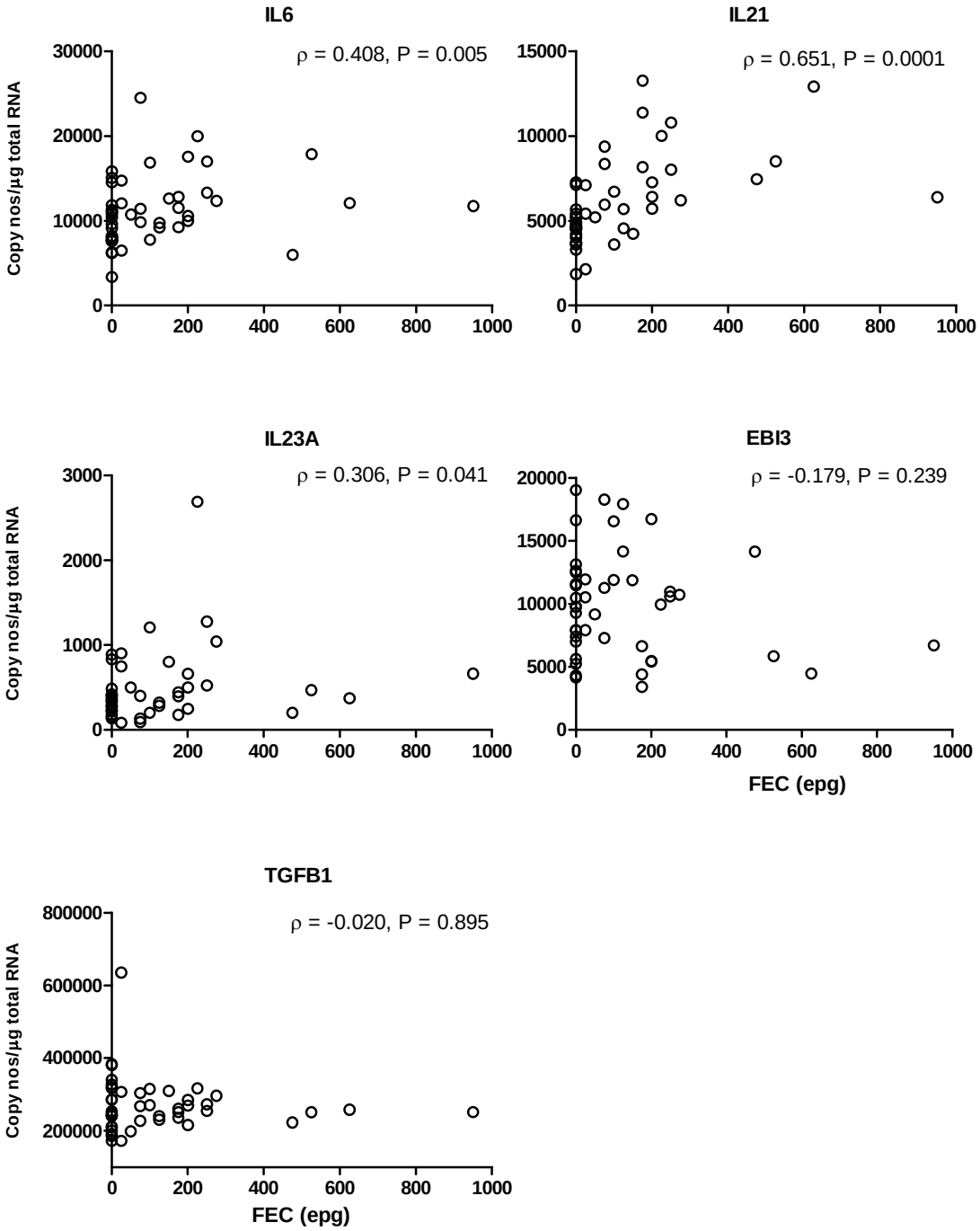


Figure 5.3 Correlation of gene copy numbers and faecal egg count (FEC) in the abomasal lymph node of 45 infected lambs. There was significant positive correlation between FEC and expression of IL6, IL21 and IL23A.

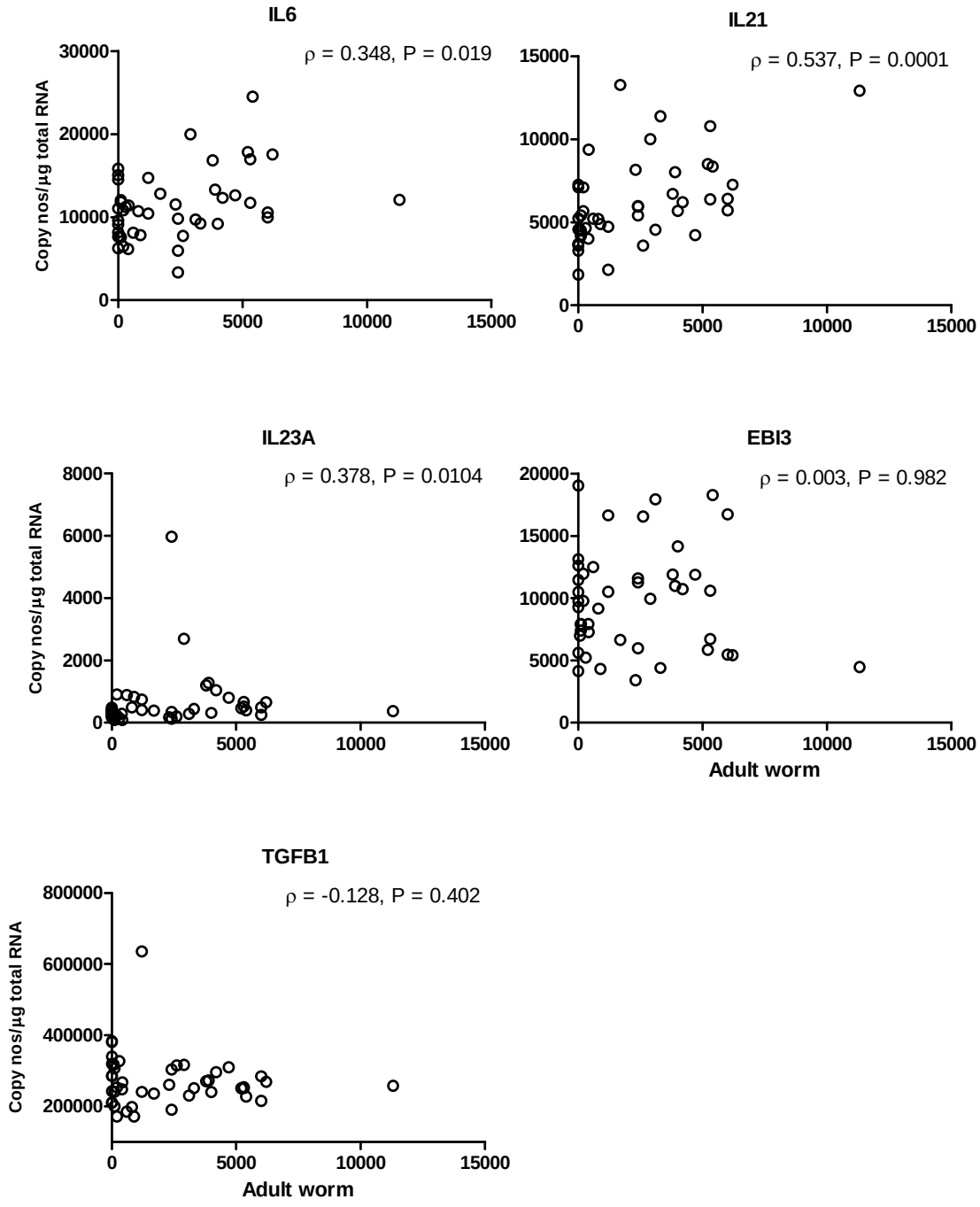


Figure 5.4 Correlation of gene copy numbers and adult worm count in the abomasal lymph node of 45 infected lambs. Significant positive correlation was found between adult worm count and expression of IL6, IL21 and IL23A .

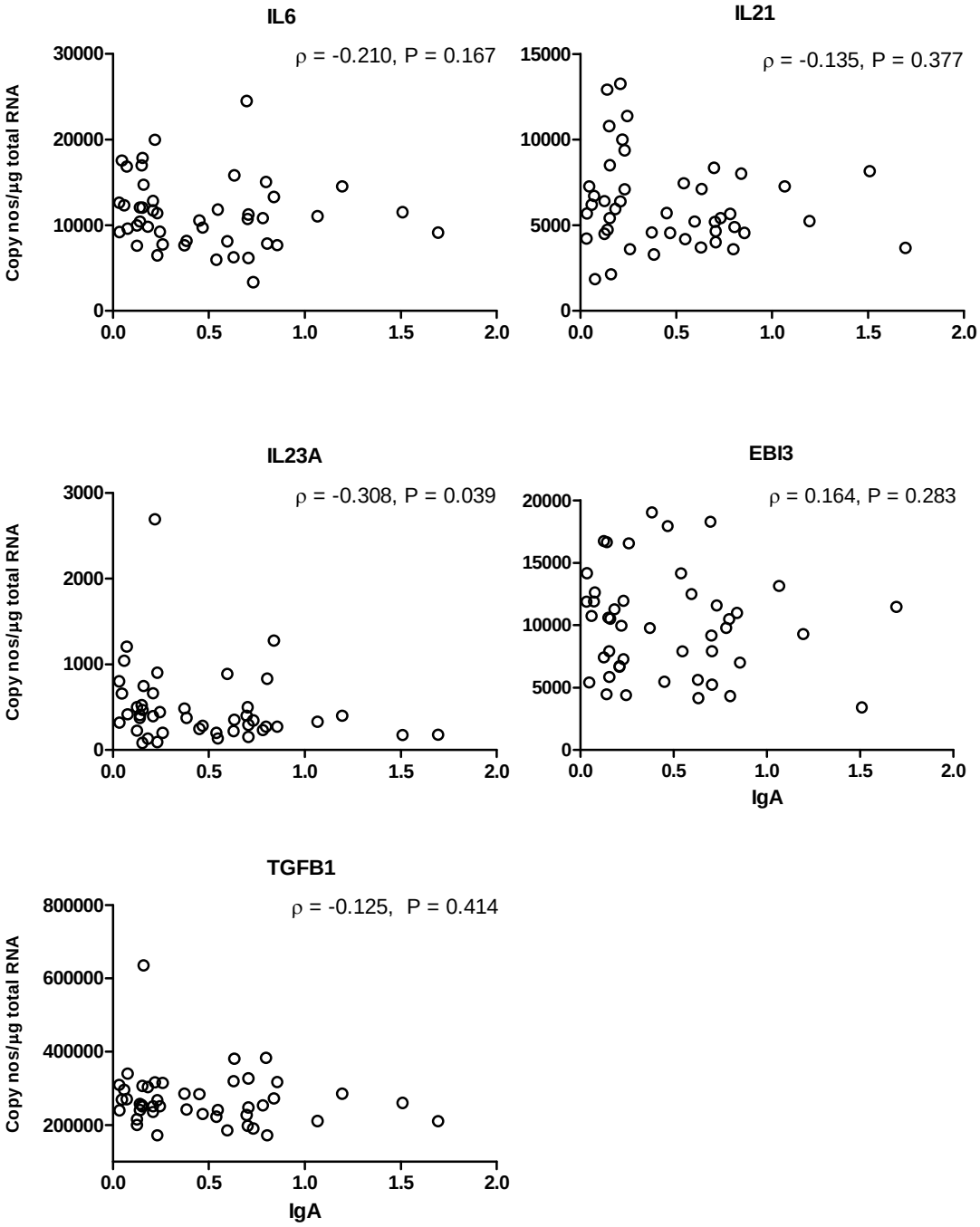


Figure 5.5 Correlation of gene copy numbers and IgA in the abomasal lymph node of 45 infected lambs. IgA is relative concentration based on the standard (Beraldi *et al*, 2008). Significant negative correlation was found between IgA and expression of IL23A .

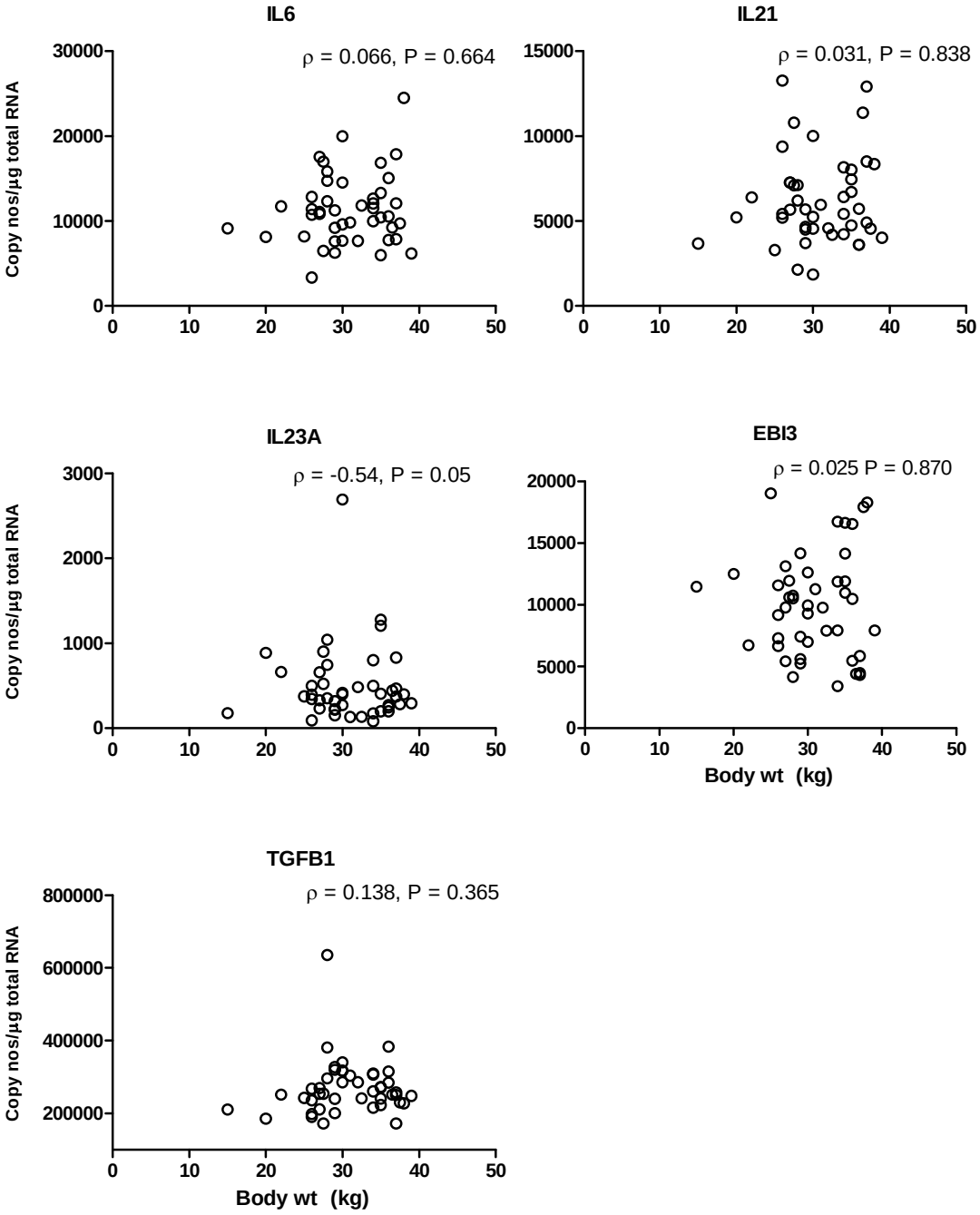


Figure 5.6 Correlation of gene copy numbers and body weight (BW) in the ALN of 45 infected lambs. No significant correlation was found between body weight and expression of any of the gene transcripts.

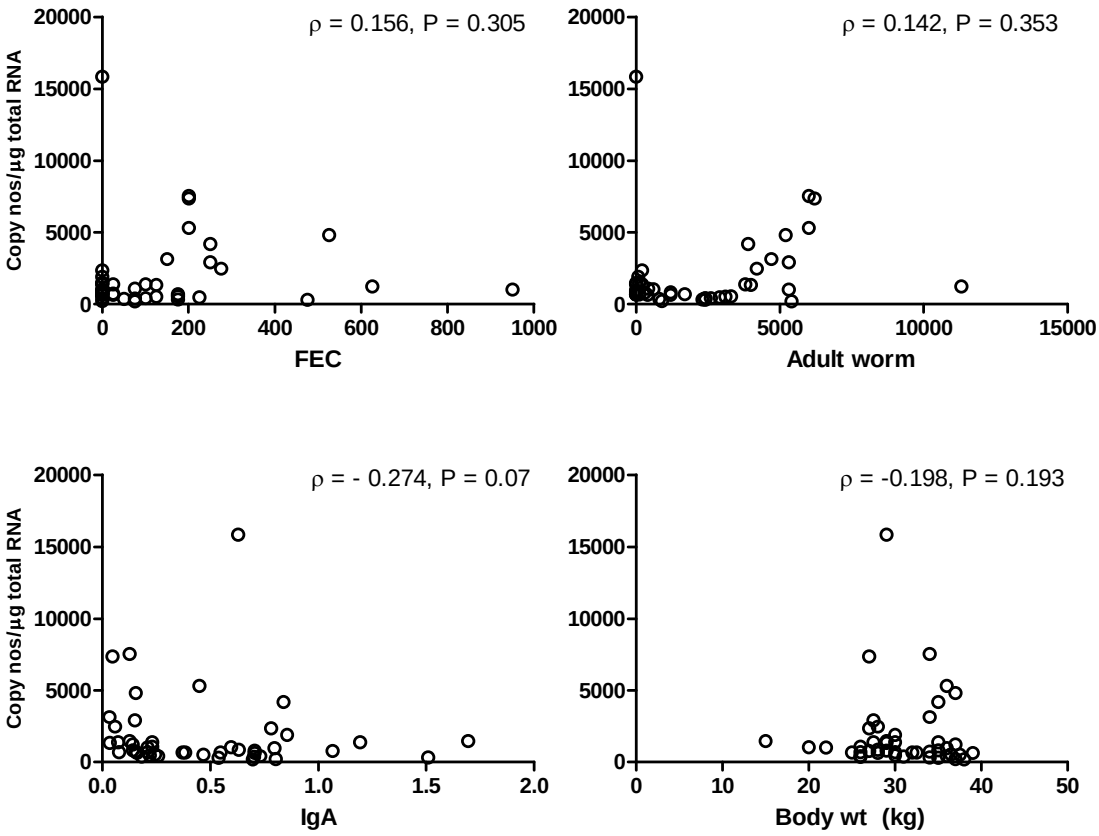


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Figure 5.7 Correlation of IL6 copy numbers with phenotype data in the ABM of 45 infected lambs. No significant correlation was found between IL6 expression and any of the phenotype parameters

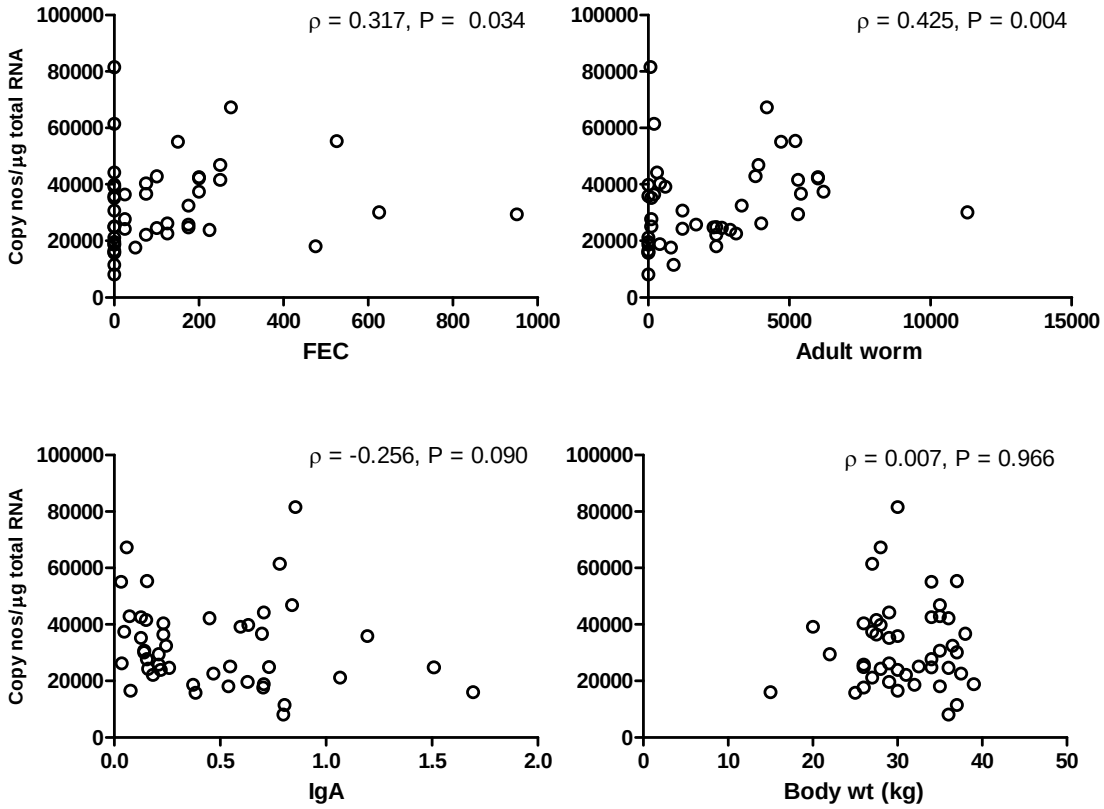


Figure 5.8 Correlation of TGFB1 copy numbers with phenotype data in the ABM of 45 infected lambs. There was significant positive correlation between TGFB1 expression and faecal egg count and adult worms.

5.4 Summary and conclusion

Absolute quantification of cytokine transcript copy numbers were generated in 55 lambs for five cytokine transcripts in the abomasal lymph node and two cytokine transcripts in the abomasal mucosa. Group comparisons of copy numbers in the abomasal lymph node revealed that IL6, IL21, and IL23A had significantly higher transcript levels in susceptible than resistant lambs. IL6 was also significantly raised in susceptible than in the intermediate group. Animals in the control group had increased expression of IL6 and IL21 compared with the intermediate and susceptible groups. No significant differences were found with any group comparisons for EBI3 and TGFB1. Comparisons between groups on copy number measurements in the abomasal mucosa revealed a significantly higher level of TGFB1 in susceptible compared with resistant lambs. No significant differences were found between groups for IL6.

There was significant positive correlation of IL6, IL21, and IL23A expression in the abomasal lymph node with FEC and adult worm count. IL23A was negatively correlated with IgA. Body weight was not significantly correlated with any of the gene transcripts. There were no significant correlations found in EBI3 and TGFβ1 with any of the phenotypic parameters. Significant positive correlations were seen between TGFB1 copy numbers in the abomasal mucosa with adult worm count and FEC.

Chapter 6 Identification of genetic markers for resistance

6.1 Overview

It is well established that much of the variation of resistance to gastrointestinal parasitic nematodes in sheep is under genetic control (Bishop and Stear, 2003; Gray, 1997; Ingham *et al.*, 2008; Stear *et al.*, 1997; Windon, 1996). The ultimate aim of the project is to enable the selection of lambs resistant to *T. circumcincta*. The current criteria for selection are a number of indicator traits, primarily FEC(Davies *et al.*, 2005; Gill, 1991; Stear *et al.*, 2006a) and pathological parameters, including eosinophilia (Stear *et al.*, 2002) and IgA antibody levels (Davies *et al.*, 2006; Stear *et al.*, 1997; Strain *et al.*, 2002). However, such phenotypic markers are quite difficult to establish as it necessitates challenge infection with the parasite. This process does not only compromise the animal's productivity but also requires longer time to evaluate the indicator trait (Davies *et al.*, 2005). In addition, phenotypic parameters may be influenced by several factors such as time after infection, season, age, and nutritional status as reviewed by Stear *et al.* (2007b) and therefore would require careful assessment of the index of trait heritability.

Single nucleotide polymorphisms could be used as genetic markers for resistance that can be validated with phenotype markers as mutations in particular loci of a gene may alter the gene product and gene expression or regulation. Examples are IFNG (Coltman *et al.*, 2001b; Matika *et al.*, 2011) and IL-4 (Benavides *et al.*, 2009) genes where polymorphism conferring increased resistance to gastrointestinal nematode parasites has been located.

Genetically-controlled resistance of sheep has an immunological basis (Gill, 1991), thus differential expression of genes involved in the polarization of an immune

response is useful in determining candidate genes as markers. The strategy used in this study was to identify candidate genes as markers based on their immunological association with resistance.

In this section, we evaluated two parameters in identifying genetic markers that are associated with resistance to *T. circumcincta*. First, we aimed to detect SNPs in IL21 gene and its receptor IL21R. We have shown that IL21, IL6 and IL23A are differentially expressed in resistant and susceptible lambs suggesting a link between susceptibility and the activation of the inflammatory TH17 T cell subset. We focused our interest on IL21 which plays a major role in the maintenance of TH17 response cells (Korn *et al.*, 2009). IL21 was up-regulated by > two-fold in susceptible lambs and was also significantly positively correlated with both adult worm numbers and faecal egg count. It was hypothesized that this response has a genetic basis and that polymorphisms (SNPs) of the IL21 and/or, IL21R genes are linked to phenotypic parameters of susceptibility. If so, these could be utilized as selectable genetic marker for resistance to *T. circumcincta.*

Initially, I generated the ovine sequence for the coding regions of IL21 and IL21R genes prior to identification of any sequence variations. High resolution melt analysis was used to identify these sequence variations in IL21 and IL21R. This technique, which is used for mutation scanning and genotyping, allows characterization of the DNA based on the transition from double stranded to single stranded DNA with increasing temperature (Erali *et al.*, 2008). DNA melt is influenced by the amplicon size and the base constituent which is reflected in the

temperature shift making HRM analysis a powerful tool that is sensitive enough to detect even a single base mutation (White and Potts, 2006).

The second part dealt with determining if the allele *Ovar-DRB1*0203*, which has been linked with resistance to *T. circumcincta,* could be identified in resistant lambs used in the study. This part was not included in the original thesis plan but I became interested with the recent finding on the association of this allele (Hassan *et al.*, 2011) with resistance to *T. circumcincta.* Since I was doing the SNP analysis during that time, I decided to maximize the use of my gDNA samples,

*Ovar-DRB1*0203* is one of theloci in DRB1 allele MHC Class II. Several allele types have been associated with the MHC Class II region owing to its important role in antigen presentation and consequent T cell activation. Of the many MHC Class II genes that have been characterized in ruminants only DRB1 has been found to display extensive polymorphism in sheep (Dutia *et al.*, 1994; Schwaiger *et al.*, 1995). Several alleles have also been identified within this locus and more recently it has been reported that Suffolk sheep that carry *Ovar DRB*0203* have increased resistance to natural *T. circumcincta* infection compared to those expressing different alleles (Hassan *et al.*, 2011). It was also revealed that substitution of the most common allele with *Ovar DRB*0203* has reduced FEC to 58-fold in six-month-old lambs following natural, predominantly *T. circumcincta* infection (Schwaiger *et al.*, 1995). Based on the phenotype data (Beraldi *et al.*, 2008), FEC and adult worm count are significantly higher in susceptible lambs compared to their resistant counterpart. On this premise, we aimed to identify if *Ovar DRB*0203* (*1101) within sheep MHC Class II is expressed in resistant lambs.

6.2 SNP analysis in IL21 and IL21R

6.2.1 Extraction of genomic DNA from blood of lambs

Genomic DNA was extracted from blood of the ten most resistant (lowest adult worm count and FEC) and ten most susceptible animals (highest adult worm count and FEC). This was used as a template for identifying SNPs by HRM analysis. The concentration of extracted DNA is presented in Table 6.1. The extracted DNA was generally of high purity with 260/280 absorbance ratio that ranged from 1.73-1.91.

6.2.2 PCR of IL21 and IL21R fragments

The full length sequences of ovine IL21 and IL21R were not available and the bovine mRNA sequences (IL21, NM_198832.1; IL21R, NM_001193179.1) for both genes were utilized as templates to design primers for cloning the ovine genes. The generated ovine sequence was then used to design primers for HRM analysis, which require short amplicon size of 100-200 bp to increase the sensitivity of the assay. Primers used to generate the sequences are shown in Table 6.2. Standard cycling conditions were used as described in Section 1.2.3.2. Two primer sets were needed to amplify the 459-base IL21 mRNA because there was no suitable single primer set that could span its entire coding region. This was attributed to the characteristic arrangement of some bases, e.g. high G/C rich or long A/T rich areas in many regions making it difficult to design an ideal primer set

Table 6.1 DNA sample concentration used in HRM analysis

Group	Sample	DNA concentration (ng/ul)	260/280 absorbance ratio
Resistant	1	98.21	1.88
	2	88.36	1.85
	3	85.42	1.91
	4	91.05	1.86
	5	34.67	1.77
	6	27.40	1.73
	7	50.53	1.80
	8	32.3	1.85
	9	40.71	1.82
	10	34.87	1.91
Susceptible	11	84.28	1.84
	12	72.13	1.90
	13	32.14	1.84
	14	26.99	1.78
	15	83.49	1.84
	16	31.53	1.86
	17	26.61	1.86
	18	75.14	1.81
	19	33.43	1.91
	20	68.84	1.84

Table 6.2 Primers used to generate partial and full length coding region sequence of ovine IL21 and IL21R

Gene	Primer	Accession no.	Product size	Tm (ºC)
IL21	F: CGGGGAACATGGAGAGAATA	NM_198832.1	257	55
	R: TCCATTATTTGCTGACTTTAG			
	F: ACTATGTGAATGACTTGGATCC		311	55
	R: CTAGGACAGATGCTGATGAATC			
IL21R	F: CCTCTTTGGGATGTAACTGATG	NM_001193179.1	456	60
	R: CATATGTGTCATGCCAGGTG			
	F: CGACTTTGCTCCTGCTAATG		1018	58
	R: TCAGCTTCCACCAGGTCTG			
	F: ACCCAGTCGTCTTTCACACC		974	61
	R: CACACTCTGAACACCAGGCTC			

6.2.3 Amplification of cDNA 5' end of IL21

The published bovine IL21 mRNA sequence in the database consisted only of the coding region, which did not allow me to design primers that would cover the entire coding region in sheep. In order to generate these sequences, RACE was performed (Section 1.4.1.2) in one of the total RNA samples used in real time RT-qPCR (Appendix 1). The lymph node RNA sample used was selected on the basis of its purity with RIN value of 7.5 and absorbance ratio for 260/280 at 2.15 and 2.04 for 260/230. The concentration of the RNA used was 1452.47 ng/µl.

RACE allows amplification of full length 5' and 3' cDNA using known cDNA sequence. The protocol ensures that only full-length transcripts are produced by elimination of truncated messages from the amplification process. Dephosphorylated and decapped total RNA generated the mRNA into which RNA Oligo primer was ligated. This provided a known priming site for the GeneRacer™ primers after the mRNA was transcribed into cDNA. RACE-ready first strand cDNA with known priming site for 5' end was amplified using gene-specific primers at the 5' end initially and subsequently nested gene-specific primers to increase the yield and specificity of the PCR. These primer sets are presented in Table 6.3.

Table 6.3 Primers used in amplifying 5' cDNA ends of ovine IL21

Accession number	Primer (5' to 3')	Base region	Tm (°C)
NM_198832.1	Reverse 5' end		
	CGCTCACAGTGTCTCTTTACATC	193-216	72
	TCTGGAGCTGGCAGAAATTCAGG	169-191	70
	Nested reverse 5' end		
	CTCCATGTTCCCCGGCCACCG	4-24	72
	GGCCACTGTCCCAGAGAAGATGACC	45-69	69

PCR was set up for the reverse 5' end primers as described in Section 1.4.1.3. The cycling parameters used in amplifying 5' ends of IL21 are as follows:

94ºC	2 min	1 cycle
94ºC 72ºC	30 sec 30 sec	5 cycles
94ºC 70ºC	30 sec 30 sec	5 cycles
94ºC 65ºC 68ºC	30 sec 30 sec 30 sec	20 cycles
68ºC	10 min	1 cycle

Two primer sets were tested to produce the untranslated sequences in the 5' end of IL21 using RACE protocol. If the sequence for untranslated regions of the 5' end was produced then we could have subsequently worked on the 3' end to complete the coding regions of IL21. However, neither of these sets, including the nested primers used, generated the target product. A faint smear on the gel was seen for the PCR product of one of the primer sets, which was cloned and sequenced as described in Section 1.2.4 but did not match the target sequence. These observations may be attributed to the characteristics of the bases comprising the untranslated regions that would require highly specific and efficient thermo-cycling conditions. Optimization of the annealing conditions may have improved the results but this was not performed due to time limitations.

The main purpose in generating the ovine sequences of IL21 was to provide a template from which sequences of possible ovine SNPs from resistant and/or

susceptible lambs will be aligned to. The sequences at the 31 bases in the 5' end and the 22 bases in the 3'end were not successfully generated in the 459-base long coding region of IL21. Nevertheless, the rest of the sequences within the coding region were obtained which could be utilized in identifying possible SNPs that span those areas.

6.2.4 Generation of coding sequences for ovine IL21 and IL21R

Two batches of PCR were performed for ovine IL21 and IL21R sequencing primers (Table 6.2) using lymph node cDNA templates sourced from two different RT reactions. This was done to generate high quality consensus sequence of transcripts with minimal ambiguity. Products were cloned and sequenced as described in Section 1.2.4. At least three independent sequences from each primer sets of IL21 and IL21R in both PCR runs were generated. Sequences were aligned with the coding regions of the bovine sequence in CLC Sequence Viewer v6.5.2 to generate a consensus. Each pair of overlapping fragments of IL21 and IL21R consensus sequences were aligned in NeedleMan-Wunsch global alignment application implemented in Jemboss v 1.5 (Carver and Bleasby, 2003). The pairs were then joined together to generate the full length sequence using Merger software in Jemboss. Partial and full length sequences of IL21 and IL21R respectively were aligned with related species using ClustalW2. The created alignments, along with their alignment scores are shown in Figures 6.1 and 6.2. Ovine IL21 and IL21R transcripts were closely related with the bovine species with matched nucleotide bases of 97% and 94% respectively.

6.2.5 Genomic organization of IL21 and IL21R

The ovine IL21 and IL21R mRNA sequences were aligned with the ovine genome using the database in sheep genome Oar v2.0 (http://www.livestockgenomics.csiro.au/blast/) genome sequences to identify their genomic organizations. IL21 was found in chromosome 17 and consists of five exons and four introns as illustrated in Fig 6.3. IL21R was located in chromosome 24 with a total of eight exons within the coding regions (Fig 6.4). Untranslated regions lie in the first exon of the 5' end and part of the last exon of the full length sequence.

```
OVINE      ---------------------------------------TGAATTTCTGCCAGCTCCAG 20
BOVINE     TTGTTGATCAGCTGAAAAACTATGTGAATGACTTGGATCCTGAATTTCTGCCAGCTCCAG 190
HUMAN      TTGTTGATCAGCTGAAAAATTATGTGAATGACTTGGTCCCTGAATTTCTGCCAGCTCCAG 238
PORCINE    CTGTTGATCAGCTGAAAAATTATGTTCATGACTTGGACCCTGAATTGCTGCCAGCTCCAG 190
MURINE     TTGTTGAACAGCTGAAAATCTATGAAAATGACTTGGATCCTGAACTTCTATCAGCTCCAC 225
                                                 **** * **. ********

OVINE      AAGATGTAAAGAGACACTGTGAGCGGTCAGCTTTTTCATGTTTTCAGAAGGTTCAACTAA 80
BOVINE     AAGATGTAAAGAGACACTGTGAGCGGTCAGCTTTTTCATGTTTTCAGAAGGTTCAACTAA 250
HUMAN      AAGATGTAGAGACAAACTGTGAGTGGTCAGCTTTTTCCTGCTTTCAGAAGGCCCAACTAA 298
PORCINE    AAGATGTACAGAGACACTGTGAGCAGTCAGCTTTTTCATGTTTTCAGAAGGTCGAACTAA 250
MURINE     AAGATGTAAAGGGGCACTGTGAGCATGCAGCTTTTGCCTGTTTTCAGAAGGCCAAACTCA 285
           ******** **. ..******** .  ******** *.** **********   ****.*

OVINE      AGTCAGCAAATAATGGAGACAACGAAAAGATAATCAACATACTAACTAAACAGCTGAAGA 140
BOVINE     AGTCAGCAAATAATGGAGACAACGAAAAGATAATCAACATATTAACTAAACAGCTGAAGA 310
HUMAN      AGTCAGCAAATACAGGAAACAATGAAAGGATAATCAATGTATCAATTAAAAAGCTGAAGA 358
PORCINE    AGTCAGCAAATACGGGAGACAATGAAAAGATAATCAATGTATTAATAAAACAGCTGAAGA 310
MURINE     AGCCATCAAACCCTGGAAACAATAAGACATTCATCATTGACCTCGTGGCCCAGCTCAGGA 345
           ** ** **** .. ***.**** .*.* .:*.****: .:.  ..  ....**** *.**

OVINE      GGAAACTGCCTCCCACAAATGCAGGGAGAAGACAGAAACATGAACTAACATGTCCTTCTT 200
BOVINE     GGAAACTACCTGCCACAAATACAGGGAGAAGACAGAAACATGAAGTAACATGTCCTTCTT 370
HUMAN      GGAAACCACCTTCCACAAATGCAGGGAGAAGACAGAAACACAGACTAACATGCCCTTCAT 418
PORCINE    GGAAACTACCTCCCACAAATGCAGGGAGAAGACAGAAACATGGGCTAACATGTCCTACAT 370
MURINE     GGAGGCTGCCTGCCAGGAGGGGAGGAAAGAAACAGAAGCACATAGCTAAATGCCCTTCCT 405
           ***..* .*** *** .*. . ***.*..*.******.** . .  :*.*** ***:* *

OVINE      GTGATTCTTATGAGAAAAAGCCACCCAAGGAATACCTAGAAAGACTGAAATCGCTCATCC 260
BOVINE     GTGATTCTTATGAGAAAAAACCACCCAAGGAATACCTAGAAAGACTGAAATCACTCATCC 430
HUMAN      GTGATTCTTATGAGAAAAAACCACCCAAAGAATTCCTAGAAAGATTCAAATCACTTCTCC 478
PORCINE    GTGATTCGTATGAGAAAAAACCAATCAAAGAATTCCTAGAAAGACTGAAATCGCTCATCC 430
MURINE     GTGATTCGTATGAGAAAAGGACACCCAAAGAATTCCTAGAAAGACTAAAATGGCTCCTTC 465
           ******* **********...**. ***.****:********** * **** .** .* *

OVINE      AAAAG------------------------------------------------------- 265
BOVINE     AAAAG------------------------------------------------------- 435
HUMAN      AAAAGGTATCTACCTTAAGTTTCATTTGATTTTCTGCTTTATCTTTACCTATCCAGATTT 538
PORCINE    AAAAG------------------------------------------------------- 435
MURINE     AAAAG------------------------------------------------------- 470
           *****

OVINE      ----------------------------------AT---------------------- 267
BOVINE     ----------------------------------ATGATTCATCAGCATCTGTCCTAG- 459
HUMAN      GCTTCTTAGTTACTCACGGTATACTATTTCCACAGATGATTCATCAGCATCTGTCCTCTA 598
PORCINE    ----------------------------------ATGATTCATCAGCATCTGTCCTAG- 459
MURINE     ----------------------------------ATGATTCATCAGCATCTCTCCTAGA 495
                                             **
```

Figure 6.1 Alignment of sheep IL21 nucleotide sequence with cattle (NM_198832.1), human (NM_001207006.1), pig (NM_214415.1), and mouse (NM_021782.2) sequences; with alignment scores against ovine transcripts of 97 %, 86 %, 91 %, and 72 % respectively. (*) indicates identical nucleotide bases in all sequences

```
OVINE      CCCCAATACCAAGCCCAGCTGCCACCCCTGGGAGACCACCGGACTGCCCGGAGCCCAGAG 60
BOVINE     ------------------------------------------------------------
EQUINE     ------------------------------------------------------------
HUMAN      ----ATGAGCTGTCGCTGCATCTTTCTCATG-----------AAGCACGGGGAACGGGT 44
MURINE     ------------------------------------------------------------


OVINE      AAAATGGATTTCTGAGAAAGAAGTAGAACAGCAGGCTCCGGGAGGCAGCATGCCGTGTGC 120
BOVINE     ----------------------------------------------ATGCCGTGTGC 11
EQUINE     ----------------------------------------------ATGCTGTGTGG 11
HUMAN      CGGATG--------------------------GCCCGTGGGAGTCAGCATGCCGCGTGG 77
MURINE     ----------------------------------------------ATGCCCCGGGG 11
                                                           ****   * *

OVINE      CTGGGCTGCGACTTTGCTCCTGCTAATGCTCCAGGGAACCTGGGGCTGCTCCAACCTCGT 180
BOVINE     CTGGGCTGCGACTTTGCTCCTGCTAATGCTCCAGGGAACCTGGGGCTGCTCCAACCTCGT 71
EQUINE     CTGGGCTATCCCTTGGCTCCTGCTGATGCTCCAGGGTGCCTGGGGCTGCTCAGACCTTGT 71
HUMAN      CTGGGCCGCCCCCTTGCTCCTGCTGCTGCTCCAGGGAGGCTGGGGCTGCCCCGACCTCGT 137
MURINE     CCCAGTGGCTGCCTTACTCCTGCTGATTCTCCATGGAGCTTGGAGCTGCCTGGACCTCAC 71
           *  .*  .   * * .********..* ***** **:.  ***.*****   .**** .

OVINE      CTGCTACACCGATTACATCGAGACTATCACCTGCATCCTGGAGACATGGGCCGGGCACCC 240
BOVINE     CTGCTACACCGATTACATCGAGACTATCACCTGCATCCTGGACACATGGGCCAGGCACCC 131
EQUINE     CTGCTACACCGATTACCTCCAGACGGTCACCTGTATCCTGGAGACATGGACCCTCCACCC 131
HUMAN      CTGCTACACCGATTACCTCCAGACGGTCATCTGCATCCTGGAAATGTGGAACCTCCACCC 197
MURINE     TTGCTACACTGACTACCTCTGGACCATCACCTGTGTCCTGGAGACACGGAGCCCCAACCC 131
            ******** ** ***.** .*** .*** *** .******* * . **. *   .****

OVINE      CGACTCGCTCACCCTCACCTGGCATGACACATTTGAAGAACTGGAGGATGAAGTCACCTC 300
BOVINE     CGACTCGCTCACCCTCACCTGGCATGACACATATGGAGAACTGGAGGATGAAGTCACCTC 191
EQUINE     TGGCACGCTCGTCCTCGCCTGGCAAGACTCGTACGGAGAACTGGAGGATGAGGTCACCTC 191
HUMAN      CAGCACGCTCACCCTTACCTGGCAAGACCAGTATGAAGAGCTGAAGGACGAGGCCACCTC 257
MURINE     CAGCATACTCAGTCTCACCTGGCAAGATGAATATGAGGAACTTCAGGACCAAGAGACCTT 191
            ..*: .***.  ** .*******:**  ..*: *..**.**  ****  *.*  ****

OVINE      CTGCAGCCTCCTCCAGTCCACCCACAATGCCACCCATGCGGAGTACACGTGCCACATGAA 360
BOVINE     CTGCAGCCTCCTCCGGTCCACCCACAACGCCACCCACGTGGAGTACACGTGCCACATGAA 251
EQUINE     CTGCAGCCTCGGCCGCTCCACCCACAACGCCACGCACACAGAGTACACGTGCCACATGGA 251
HUMAN      CTGCAGCCTCCACAGGTCGGCCCACAATGCCACGCATGCCACCTACACCTGCCACATGGA 317
MURINE     CTGCAGCCTACACAGGTCTGGCCACAACACCACACATATATGGTACACGTGCCATATGCG 251
           *********.  *.. ** . ****** .**** ** .     ***** ***** *** .

OVINE      AGTGTTCTCCCTCGTGGCTGACGACTTCTTCAATGTAAGCATGACAGACCCATCCGGCAA 420
BOVINE     TGTGTTCCGCCTCATGGCTGACGACTTCTTCAATGTAAGCATGACAGACCCATCCGGCAA 311
EQUINE     CGTGTTCCCCTTCATGGCCGACGACATCTTCACTGTCAACATGACAGACCAGTCTGGCAA 311
HUMAN      TGTATTCCACTTCATGGCCGACGACATTTTCAGTGTCAACATCACAGACCAGTCTGGCAA 377
MURINE     CTTGTCTCAATTCCTGTCCGATGAAGTTTTCATTGTCAATGTGACGGACCAGTCTGGCAA 311
             *.*    . ** ** * ** **. * **** ***.*. .* **.****..** *****

OVINE      CTACTCCAAGGAGTGTGGCAGCTTTATGGTGGCTGCGAGCATCAAGCCATCTCCCCCTTT 480
BOVINE     CTACTCCCAGGAGTGTGGCAGCTTTATAGTGGCTGCGAGCATCAAGCCATCTCCCCCTTT 371
EQUINE     CCACTCCCAGGAGTGCGGCAGCTTTGTCCTAGCGAAGAGCATCAAGCCATCTCCCCCTTT 371
HUMAN      CTACTCCCAGGAGTGTGGCAGCTTTCTCCTGGCTGAGAGCATCAAGCCGGCTCCCCCTTT 437
MURINE     CAACTCCCAAGAGTGTGGCAGCTTTGTCCTGGCTGAGAGCATCAAACCAGCTCCCCCCTT 371
           * *****.*.***** ********* *  *.** ..*********.**. ******* **
```

Figure 6.2 Alignment of sheep IL21R nucleotide sequence with cattle (NM_001193179.1), horse (XM_001496989.1), human (NM_181079.4), and mouse (NM_021887.2) sequences; with alignment scores against ovine transcripts of 94 %, 79 %, 74 %, and 72 % respectively. (*) indicates identical nucleotide bases in all sequences

```
OVINE      CAACGTGACCGTGAGCTTCTCCGGAAATTATAACATCTCCTGGAGTTCCAGTTCCAGTTC 540
BOVINE     CAACGTGACCGTGAACTTCTCCGGATATTATAATGTCTCTTGGAGTTCCAGTTCCAGTTT 431
EQUINE     CAACGTGACCGTGACCTTCTCGGGACTTTACAACATCTCCTGGAGCTCCAGTTACGATTC 431
HUMAN      CAACGTGACTGTGACCTTCTCAGGACAGTATAATATCTCCTGGCGCTCAGATTACGAAGA 497
MURINE     GAACGTGACTGTGGCCTTCTCAGGACGCTATGATATCTCCTGGGACTCAGCTTATGACGA 431
            ******** ***. ****** ***   ** .* .**** *** . **.. **. ..

OVINE      CAGTCCCAGGGACCTTGTCTACGCCCTGAAGGACAAACTTCAGTATGAGCTGCGGTACAG 600
BOVINE     CAGTCCCAGTGACCGTGTCTATGCACTGAAGGACAAACTTCAGTATGAGCTGCGGTACAG 491
EQUINE     CT-----------------ACGGGCTGCAGGGCAAGCTTCAGTACGAGCTGCAGTACAA 473
HUMAN      CCCTGCCT---------TCTACATGCTGAAGGGCAAGCTTCAGTATGAGCTGCAGTACAG 548
MURINE     ACCCTCCA---------ACTACGTGCTGAGGGGCAAGCTACAATATGAGCTGCAGTATCG 482
           .                  * .  ***..**.***.**:**.** *******.*** ..

OVINE      GAAGGTTGGAGAACCCTGGGCTCAGAGTCCAGGGAGAAAGCTGATCTCAGAGGATTCCAG 660
BOVINE     GAAGGTTGGAGAGCCCTGGGCTCAGAGTCCAGGGAGAAAGCTGATCTCAGAGGATTCCAG 551
EQUINE     GATGCGGGGAGATCCCTGGGCTCTGAGGCCACAGAAAAGGCTGATCTCGGTGGACTCGAG 533
HUMAN      GAACCGGGGAGACCCCTGGGCTGTGAGTCCGAGGAGAAAGCTGATCTCAGTGGACTCAAG 608
MURINE     GAACCTCAGAGACCCCTATGCTGTGAGGCCGGTGACCAAGCTGATCTCAGTGGACTCAAG 542
           **:    .**** ****. *** :*** **.  ** .*.*********.*:*** ** **

OVINE      AAGCGTCCTTCTCCTCCCTTTGGAGTTCCACAGCGGCTCAAACTACGAGCTGCAGGTGCG 720
BOVINE     AAGCGTCTTTCTCCTCCCTTTGGAGTTCCACAGTGGGTCAAGCTACGAGCTGCAGGTGCG 611
EQUINE     AAGCGTCTCCCTCGTTCCCTTGGAGTTCCGCGGAGACTCGAGCTACGAGCTGCAGGTGCG 593
HUMAN      AAGTGTCTCCCTCCTCCCCCTGGAGTTCCGCAAAGACTCGAGCTATGAGCTGCAGGTGCG 668
MURINE     AAACGTCTCTCTTCTCCCTGAAGAGTTCCACAAAGATTCTAGCTACCAGCTGCAGGTGCG 602
           **. ***   **  * **  :.*******.*.. *. ** *.***  *************

OVINE      GGCAGGGCCCCAGCCCGGCTCCACCTTCCAGGGCACCTGGAGCGAATGGAGTGAGCCAGT 780
BOVINE     GGCAGGGCCCCAGCCCGGCTCCACCTTCCAGGGGACCTGGAGCGAATGGAGTGACCCAGT 671
EQUINE     GGCAGGGCCCCAGCCTGGCTCCTCCTTTGAGGGGACGTGGAGCGAGTGGAGTGAGCCAGT 653
HUMAN      GGCAGGGCCCATGCCTGGCTCCTCCTACCAGGGGACCTGGAGTGAATGGAGTGACCCGGT 728
MURINE     GGCAGCGCCTCAGCCAGGCACTTCATTCAGGGGGACCTGGAGTGAGTGGAGTGACCCCGT 662
           ***** *** .:*** ***:* :*.*:  .*** ** ***** **.******** ** **

OVINE      CGTCTTTCACACCCAGCCGGAAGAGAGAAAGGGAGACCTGTATCTTCACCTGGTTCCCAT 840
BOVINE     CGTCTTTCACACCCAGCCGGAAGAGAGAAAGGGAGACCTGTATCTTCACCTGGTTCCCAT 731
EQUINE     CATCTTTCAGACACAGCCAGAAGGGAGCAAGGGAGGCTGGCACTCTGACCTGCTGTACCT 713
HUMAN      CATCTTTCAGACCCAGTCAGAGGAGTTAAAGGAAGGCTGGAACCCTCACCTGCTGCTTCT 788
MURINE     CATCTTTCAGACCCAGGCTGGGGAGCCCGAGGCAGGCTGGGACCCTCACATGCTGCTGCT 722
           *.******* **.*** * *..*.*  ..*** **.*  * *   * **.** *    .*

OVINE      CCTGCTCATCCTGGTCTGCCTCATCTTTGTCTTCTTGGGCCTGAAGGTCC------CTTG 894
BOVINE     CCCGCTCATCCTGGTCTGCCTCATCTTTGTCTTCTTGGGCCTGAAGGTCC------CTTG 785
EQUINE     CCTCCTGGTCCTCGTGCCCCCCATCCTTGTCTTCTTAGGCCTGAAGATCCACCTGCCTTG 773
HUMAN      CCTCCTGCTTGTCATAGTCTTCATTCCTGCCTTCTGGAGCCTGAAGACCCATCCATTGTG 848
MURINE     CCTGGCTGTCTTGAT---CATTGTCCTGGTTTTCATGGGTCTGAAGATCCACCTGCCTTG 779
           **      *  * .*   *   .*    *  ***: ..* ******. **       **

OVINE      GAGGCCGTGGGAAAAGGTGTGGCTGCAGGTGTCCAGCCCGAAGCCCTTCTTCCAGCCCCT 954
BOVINE     GAGGCCGTGGGAAAAGGTATGGTTGCAGGTGTCCAGCCCAAAGCCCTTCTTCCAGCCCCT 845
EQUINE     GAGGCTGTGGAAGAAGGTCTGGGTACAGGTGCCCAGCCCAGAGCGGTTCTTTCAGCCCCT 833
HUMAN      GAGGCTATGGAAGAAGATATGG--GCCG-TCCCCAGCCCTGAGCGGTTCTTCATGCCCCT 905
MURINE     GAGGCTATGGAAAAAGATATGGGCACCAGTGCCCACCCCTGAGAGTTTCTTCCAGCCCCT 839
           ***** .***.*.***.* ***  .*.. *  *** *** .**.  ***** .:******

OVINE      GTACGTGGGCCACAGCGGAGACTTCAAGAAATGGGTGGGCACACCCTTCACTGCCTCCAG 1014
BOVINE     GTACGTGGGCCACAGCGGAGACTTCAAGAAATGGGTGGGCACACCCTTCACTGCCTCCAG 905
EQUINE     GTACATGGGCCACAGCGGAGACTTCAAGAAATGGGTGGGCACGCCCATCACCCCCGCCAG 893
HUMAN      GTACAAGGGCTGCAGCGGAGACTTCAAGAAATGGGTGGGTGCACCCTTCACTGGCTCCAG 965
MURINE     GTACAGGGAGCACAGCGGGAACTTCAAGAAATGGGTTAATACCCCTTTCACGGCCTCCAG 899
           ****. **.  .******..*************** .. .* ** :****   * ****
```

Figure 6.2 cont'n.

```
OVINE      CCTGGAGCTGAGACCCTGGAGCCCCGGGGTACACTTGGCCCTGGAGATGTGCAGCCCGTG 1074
BOVINE     CCTGGAGCTGAGGCCCTGGAGCCCAGGGGTGCACTTGGCCCTTGAGATGTGCAGCCCATG 965
EQUINE     CCTGGAGCTGGGACTCTGGGCTGCGGGGACACCCTCGTCCCTGGAGGTGTACAGCTGCTG 953
HUMAN      CCTGGAGCTGGGACCCTGGAGCCCAGAGGTGCCCTCCACCCTGGAGGTGTACAGCTGCCA 1025
MURINE     CATAGAGTTGGTGCCACAGAGTTCCACAACAACATCAGCCTT--------ACATCTG--- 948
           *.*.*** **. .* . .*.   * . .. ....*   ** *        .** *

OVINE      CCCACCGCAGGGTACAGCCAAGGGTCTGGTGCCCACAGAGCTGCCGGAACCCACAGAACT 1134
BOVINE     CCCACCGCAGGGTGTAGTCAAGGGGCTGGTGCCCACGGAGCTGCCGGAGCCCACAGACCT 1025
EQUINE     CCCACCACAGAGTGTGCCCAAGGGGCGGGAGCCCACGTCGCTGCCGGAGCTGGCAGACCT 1013
HUMAN      CCCACCACGGAGCCCGGCCAAGAGGCTGCAGCTCACGGAGCTACAAGAACCAGCAGAGCT 1085
MURINE     ----TCATTGTATCCAGCCAAGGAGAAGAAGTTCCCGGGGCTGCCGGGTCTGGAAGAGCA 1004
                *.  * .   .  ****.. . * :*  *.*.  ***.*..*. *  ..*** *:

OVINE      GGTGGAAGCTGACGGGGTGCCTGAGCCGGGCTCCTGGGGCCCAGTGCCCTCCACTGCAGG 1194
BOVINE     GGTGGAAGCTGACGGAGTGCCTGAGCCGGGCTCCTGGGGCCCAGTGCCCTCCACTGCCGG 1085
EQUINE     GGTAGAAGCCGACGGAGTGCAGGAGCCAGGCTCCTGGGGCCCGGCCCCCTCCATGGCCAG 1073
HUMAN      GGTGGAGTCTGACGGTGTGCCCAAGCCCAGCTTCTGG---CCG---------ACAGCCCA 1133
MURINE     ACTGGAGTGTGATGGAATGTCTGAGCCTGGTCACTGGTGCATAATCCCCTTGGCAGCTGG 1064
           . *.**.   ** ** .** . .**** .*   ****   . .         .  **  .

OVINE      CAGCTTGGGCAGCTCGGTTTACAGCCAGGAGAAGGACCGGCTATATGGCCTGGTGTCCAT 1254
BOVINE     CAGCTTGGGCAGCTCGGTTTACAGCCAGGAGAAGGACCGGCTGTATGGCCTGGTATCCAT 1145
EQUINE     CAGCTTGGGCGGTTCAACTTACAGCCAGGAGAGGGACCGACCATACGGCCTGGTATCCAT 1133
HUMAN      GAACTCGGGGGGCTCAGCTTACAGTGAGGAGAGGGATCGGCCATACGGCCTGGTGTCCAT 1193
MURINE     CCAAGCGG---TCTCAGCCTACAGTGAGGAGAGAGACCGGCCATATGGTCTGGTGTCCAT 1121
            ...  **     **..  *****  ******..** **.* .** ** *****.*****

OVINE      CGACACGGTGACCGTGGTGGATGCAGAGGGGCTGTGTGACTGGCCCTGCACCTGTGGGGA 1314
BOVINE     CGACACGGTGACTGTGGTGGACGCGGAGGGGCTGTGTGGCTGGCCCTGCACCTGTGGGGA 1205
EQUINE     CGACACGGTGACCGTGGTGGACACAGAGGGGACGTGCGCCTGGCCCTGCACCTGTGGGGA 1193
HUMAN      TGACACAGTGACTGTGCTAGATGCAGAGGGGCCATGCACCTGGCCCTGCAGCTGTGAGGA 1253
MURINE     TGACACAGTGACTGTGGGAGATGCAGAGGGCCTGTGTGTCTGGCCCTGTAGCTGTGAGGA 1181
            *****.***** ***  .** .*.***** . .** . ********* * *****.***

OVINE      TGATGGCTACCCTGCCCTGAACCTGGACACTGGCCTGGAGCCTGGCCCAGGCACAGAGGA 1374
BOVINE     CGATGGCTACCCTGCCCTAAACCTGGACACTGGCCTGGAGCCTGGTCCGGACACAGAGGA 1265
EQUINE     CGATGGCTACCCAGCCCTGAACCTGGACACCAGCCTGGAGCCTGGCCCAGGCACCGAGGA 1253
HUMAN      TGACGGCTACCCAGCCCTGGACCTGGATGCTGGCCTGGAGCCCAGCCCAGGCCTAGAGGA 1313
MURINE     TGATGGCTATCCAGCCATGAACCTGGATGCTGGCCGAGAGTCTGGCCCTAATTCAGAGGA 1241
            ** ***** **:***.*..******* .* .*** .*** * .* ** ..   .*****

OVINE      TCCGCTCCTGAGCACGGAGGCCACAATCCTGTCTTGTGGCTGCGTCTCGGCCGGTGGCCC 1434
BOVINE     CCGGCTCCTGAGCACGGGGACCACGATCCTGTCTTGTGGCTGTGTCTCGGCCGGTGGCCC 1325
EQUINE     CCCGCTCTTGAGCACGGGGGCCACAGTCCTGTCCTGCGGCTGTGTCTCGGCCGGTGGTCC 1313
HUMAN      CCCACTCTTGGATGCAGGGACCACAGTCCTGTCCTGTGGCTGTGTCTCAGCTGGCAGCCC 1373
MURINE     TCTGCTCTTGGTCACAGACCCTGCTTTTCTGTCTTGCGGCTGTGTCTCAGGTAGTGGTCT 1301
            * .*** **.  .*.*.  * .*  * ***** ** ***** *****.*  .* .* *

OVINE      CG-----TGAGG----CCGGG---CAGCCTCCTTGACCGGCTAAGGCTGTCCCATGAGGA 1482
BOVINE     CA-----TGAGG----CCGGG---CAGCCTCCTTGACCGGCTAAGGCTGTCCCATGAGGA 1373
EQUINE     TG-----CCGGG----CTGGGGGGCAGCCTCCTTGACAGGCTAAAGCTGCCCCTTGAAGA 1364
HUMAN      TGGGCTAGGAGGGCCCCTGGG---AAGCCTCCTGGACAGACTAAAGCCACCCCTTGCAGA 1430
MURINE     CAGGCTTGGAGGCTCCCCAGG---CAGCCTACTGGACAGGTTGAGGCTGTCATTTGCAAA 1358
                 .       .**    * .**   .*****.** ***.*. *.*.** . *. :**...*

OVINE      TGAGGCAGATTGGACACCCGGGCCG----------------------------TCGGA 1512
BOVINE     TGAGGCAGATTGGACACCTGGGCCG----------------------------TCGGA 1403
EQUINE     TGAGGCAGGTTGGGTCCCAGGGTCGCCCTGGGGTGGCGG---GCGGCGAGAGGTGTCCGA 1421
HUMAN      TGGGGAGGACTGGGCTGGGGGACTGCCCTGGGGTGGCCGGTCACCTGGAGGGGTCTCAGA 1490
MURINE     GGAAGGGGACTGGACAGCAGACCCAACCTGGAGAACTGGGTCCCCAGGAGGGGGCTCTGA 1418
            *.* .*. ***.     *.   .                                ** **
```

Figure 6.2 cont'n.

```
OVINE      CAGTGAGGCAGGCTCACCTCCGGTCGGCCTGGACATGAACACGTTTGACAGCGGCTTTGC 1572
BOVINE     CAGCGAGGCGGGCTCGCCCCCGGTCGGCCTGGACATGAACACGTTTGACAGTGGCTTCGC 1463
EQUINE     CAGCGAGGCGGGCTCGCCCCCGGCCGGCCTGGACATGGACACGTTCGACAGCGGCTTCGC 1481
HUMAN      GAGTGAGGCGGGCTCACCCCTGGCCGGCCTGGATATGGACACGTTTGACAGTGGCTTTGT 1550
MURINE     GAGTGAAGCAGGTTCCCCCCCT---GGTCTGGACATGGACACATTTGACAGTGGCTTTGC 1475
            ** **.**.** ** ** *     ** ***** ***.****.** ***** ***** *

OVINE      GGACTCTGATTGGGGCAGCCCTGTGGAGAGTGACTTCAGCAGCCCCAGGGACGAGGAACC 1632
BOVINE     GGACTCTGACTGTGGCAGCCCCGTGGAGAGTGACTTCAGCAGCCCCAGGGACGAGGAACC 1523
EQUINE     GGGCTCCGACTGCGGCAGCCCTGTGGACTGTGACTTCACCAGCCCCAGGGATGAGGGGCC 1541
HUMAN      GGGCTCTGACTGCAGCAGCCCTGTGGAGTGTGACTTCACCAGCCCCGGGGACGAAGGACC 1610
MURINE     AGGTTCAGACTGTGGCAGCCCCGTGGAGACT-----------------GATGAAGGACC 1517
           .*. ** ** ** .******* ***** : *                 ** **.*..**

OVINE      CCCCAGGAGCTACCTCCGACAGTGGGTGGTCACGGCCCCTTCACCTACGCCACCGGGATC 1692
BOVINE     CCCCAGGAGCTACCTCCGACAGTGGGTGGTCATGGCCCCTCCACCTACGCCGCCGGGGTC 1583
EQUINE     CCCCCGCAGCTACCTCCGCCAGTGGGTGGTCACGGCCCCTTCTCCTGAAGGACTCGGACC 1601
HUMAN      CCCCCGGAGCTACCTCCGCCAGTGGGTGGTCATTCCTCCGCCACTTTCGAGCCCTGGACC 1670
MURINE     CCCTCGAAGCTATCTCCGCCAGTGGGTGGTCAGGACCCCTCCACCTGTGGACAGTGGAGC 1577
           *** .* ***** *****.************   * **  *:* *  .   .  **. *

OVINE      CCAGGCCAGCTAGTGATGC 1711
BOVINE     CCAGGCCAGCTAG------ 1596
EQUINE     CCAGGCCAGCTAG------ 1614
HUMAN      CCAGGCCAGCTAA------ 1683
MURINE     CCAGAGCAGCTAG------ 1590
           ****. ******.
```

Figure 6.2 cont'n.

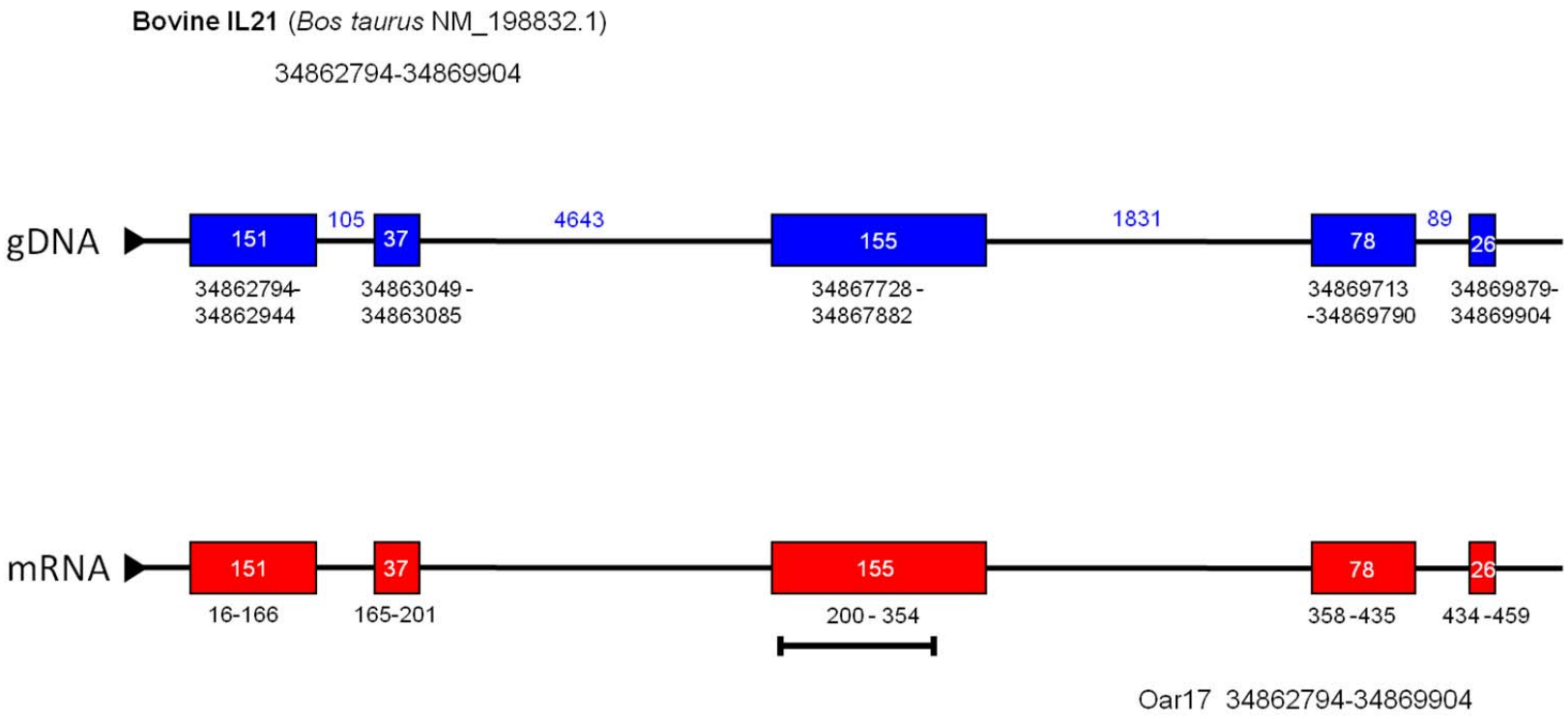


Figure 6.3 Genomic organization of ovine IL21 mRNA in relationship to bovine genomic DNA. Boxes represent exons and their corresponding size in bp. Base positions are separated by dash. Numbers in blue represent intronic bases (bp) in gDNA.

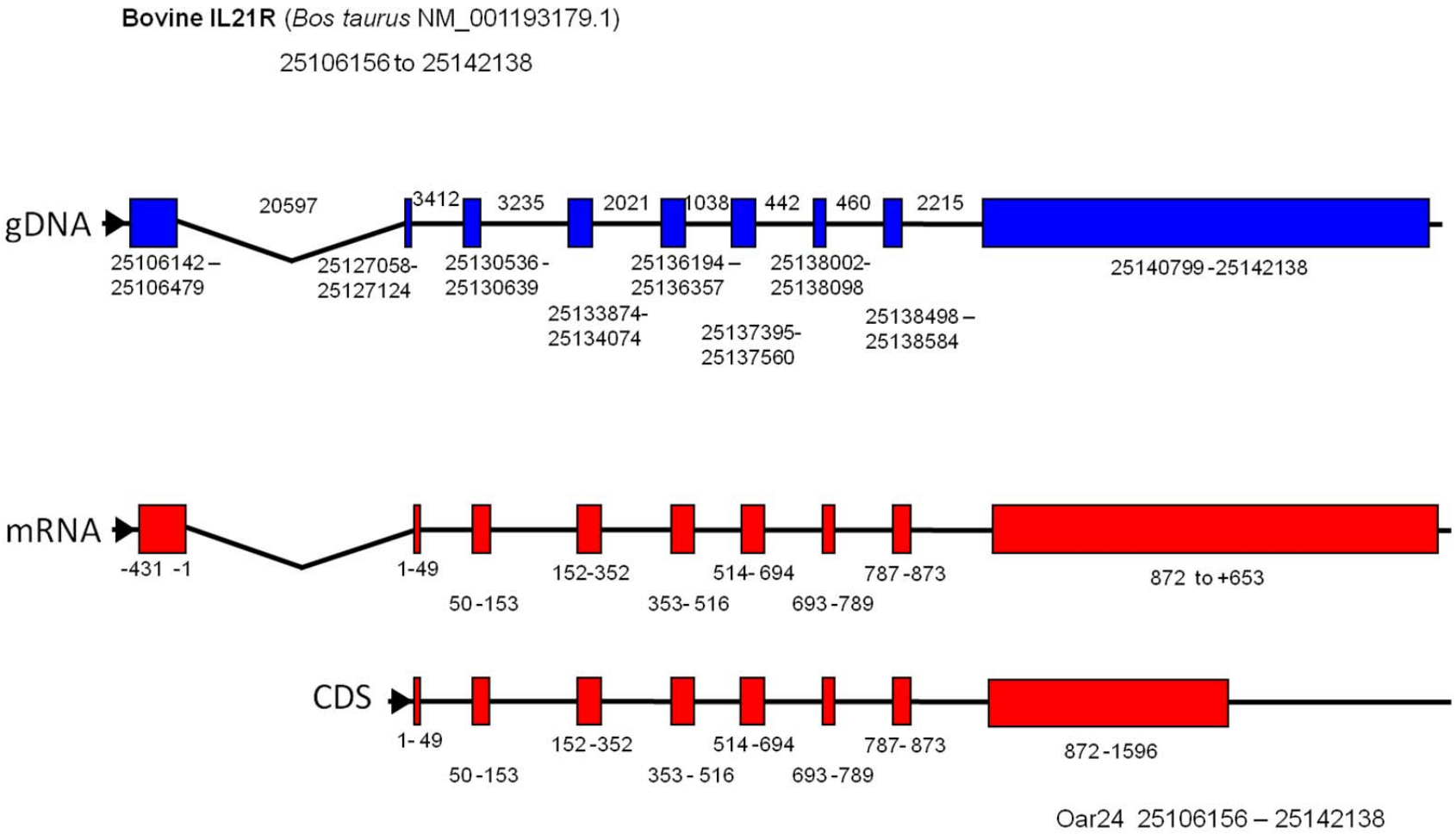


Figure 6.4 Genomic organization of the coding region (CDS) of ovine IL21R in relationship to bovine mRNA and genomic DNA. Boxes represent exons with spanning base positions separated by dash. Numbers on top are the intronic bases in the genomic DNA.

6.2.6 Optimization of HRM analysis for IL21 and IL21R fragments

Primers were to be designed to amplify all of the coding regions including the intron and exon boundaries. With limited time left to work on identifying SNPs in IL21 and IL21R, only one primer pair each for IL21 and IL21R was evaluated for HRM analysis. Primers were designed to suit the requirements for HRM as described in Section 1.4.3.1. The position of these primers relative to the coding region is depicted in Figure 6.5.

In the initial HRM-PCR runs, all set ups were performed as recommended in the Type-it® HRM™ kit as described in Section 1.4.3.2. The amount of genomic DNA template used for both genes was optimized to 4 ng per reaction. The optimized cycling conditions for the sequencing primers of IL21 and IL21R fragments are shown in Table 6.4.

Table 6.4 Primers used for HRM analysis

Primer (5' to 3')	Product size (bp)	Annealing/Elongation temperature
IL21		
F: GAGACACTGTGAGCGGTCAG	117	55ºC
R: AGTTTCCTCTTCAGCTGTTTAG		
IL21R		
F: CTTGGGCAGCTCGGTTTAC	158	57ºC
R: GCTCCAGGCCAGTGTCCAG		

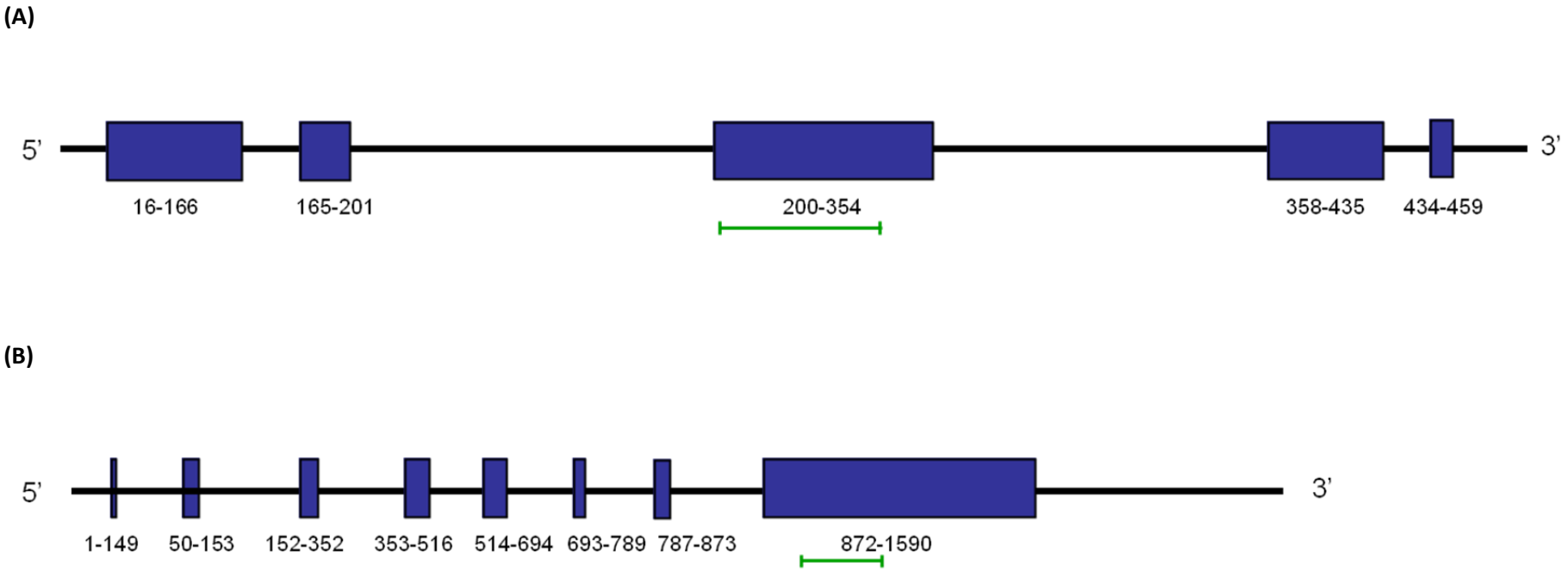


Figure 6.5 Schematic diagram of IL21(A) and IL21R (B) coding regions. Boxes represent exons with corresponding bases separated by dash that span them. Brackets indicate the length of the product amplified by the sequencing primers used in HRM analysis.

6.2.7 HRM analysis of IL21 and IL21R fragments

Fluorescence data were analysed using the tools for HRM analysis incorporated in the Rotorgene-Q software. Table 6.5 shows the comparative quantification analysis for IL21 and IL21R fragments. The mean Cq values of the HRM-PCR for IL21 was 22.66 which ranged from 21.8 to 23.5. IL21R had mean Cq values of 23.01 with a range of 21.9-23.9. The reaction efficiency for IL21 was 1.78 which was close to IL21R with 1.80. These values are consistent with the recommended Cq of < 30 % and individual reaction efficiency of < 1.4 (Nguyen-Dumont *et al.*, 2009;Vossen *et al.*, 2009). A single melt peak also confirmed a single amplified product (Figure 6.9). Figure 6.6 shows the mean cycle number at which fluorescence exceeded background (Cq) which was < 30. The range of < 23.5 was recorded across samples for IL21 fragment and < 21.9 for IL21R. All curves reached a similar plateau height.

The software automatically analysed the raw melting curve data and set the starting (pre-melt) and ending (post-melt) fluorescence signals of all data to uniform values to aid interpretation and analysis (Figure 6.7). The cursors for these two points are defaulted to the ends of the curve but these regions were manually adjusted to encompass representative baseline for the pre-melt and post-melt phases. Widening the normalization regions into the melt phase was avoided to ensure that curves normalize effectively. The two points within these regions were used to normalize fluorescence of the melt plot and data outside these regions are ignored.

Table 6.5 Comparative quantification of HRM analysis in IL21 and IL21R fragments of resistant and susceptible lambs

Sample	Group	IL21		IL21R	
		Cq	Efficiency	Cq	Efficiency
1	Resistant	23.50	1.78	23.90	1.81
2	Resistant	22.70	1.77	22.90	1.82
3	Resistant	22.80	1.82	23.20	1.83
4	Resistant	22.10	1.75	22.60	1.80
5	Resistant	22.90	1.74	23.50	1.86
6	Resistant	22.60	1.80	23.10	1.81
7	Resistant	22.60	1.79	23.20	1.80
8	Resistant	22.80	1.79	23.20	1.77
9	Resistant	22.50	1.84	22.70	1.75
10	Resistant	23.40	1.78	23.70	1.72
11	Susceptible	22.50	1.80	22.90	1.82
12	Susceptible	22.70	1.75	23.20	1.79
13	Susceptible	22.60	1.80	23.10	1.76
14	Susceptible	22.80	1.76	23.00	1.76
15	Susceptible	22.70	1.78	22.80	1.81
16	Susceptible	22.30	1.77	22.70	1.79
17	Susceptible	21.80	1.78	21.90	1.78
18	Susceptible	22.60	1.75	22.70	1.79
19	Susceptible	22.70	1.77	23.00	1.80
20	Susceptible	22.60	1.77	22.90	1.85
MEAN		**22.66**	**1.78**	**23.01**	**1.80**

HRM- high resolution melt

Cq – quantification cycle

After normalization, samples with melt temperature difference can be visualized. Figure 6.8 shows samples of IL21R gene fragment (B) with marked shift in melt temperature compared with the rest of the samples. No difference was detected across samples with IL21 (A) was detected at this stage.

The difference plot was defined by assigning one of the samples as genotype which served as a reference sample to compare all other samples against. In the example shown in Figure 6.6, all samples were compared to sample 54, which was close to the middle of the response spectrum between resistant and susceptible lambs. This was assigned as a positive control which was the same sample used as reference curve for all subsequent runs. The software groups together similar curves according to an adjustable sensitivity value. In the displays shown in Figure 6.9, the differences between melting curve profiles are readily appreciated. Sample 28 (susceptible) of IL21R (Figure 6.9B) illustrates the marked difference in the melt curve profile compared with the rest of the samples. The difference plot for sample 114 (susceptible) of IL21 fragment suggests a melt curve slightly different from the other samples (Figure 6.9A).

The choice of the reference curve sample could be arbitrarily chosen among the samples within the run for as long as this will be used in all other HRM runs with other fragments of the gene of interest. However, it is ideal that one representative sample should be assigned as reference in comparing with the melt curves of all the samples. In which case, this designated sample should be included in the HRM-PCR set up along with all samples to be tested (personal communication, Qiagen technical staff, 15 September 2011).

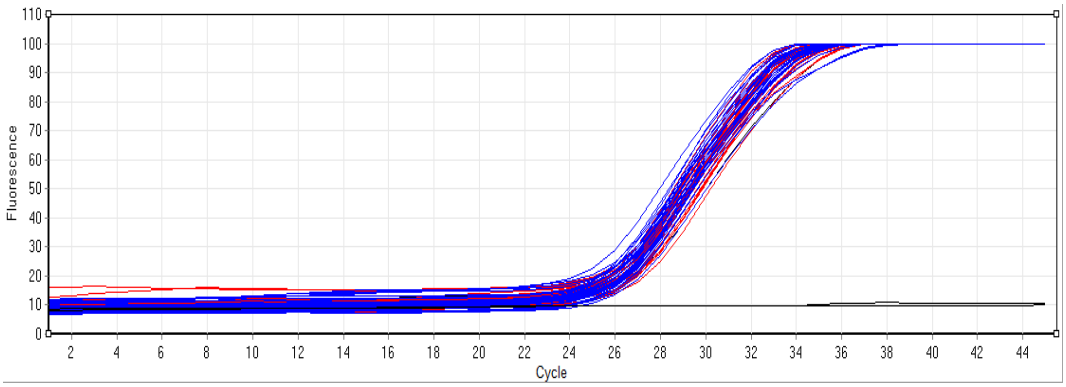


Figure 6.6 Amplification curves of samples from resistant (red) and susceptible (blue) lambs showing the quantification cycle (Cq) of <30 and the plateau height which is similar across samples. Black straight lines represent the no-template control.

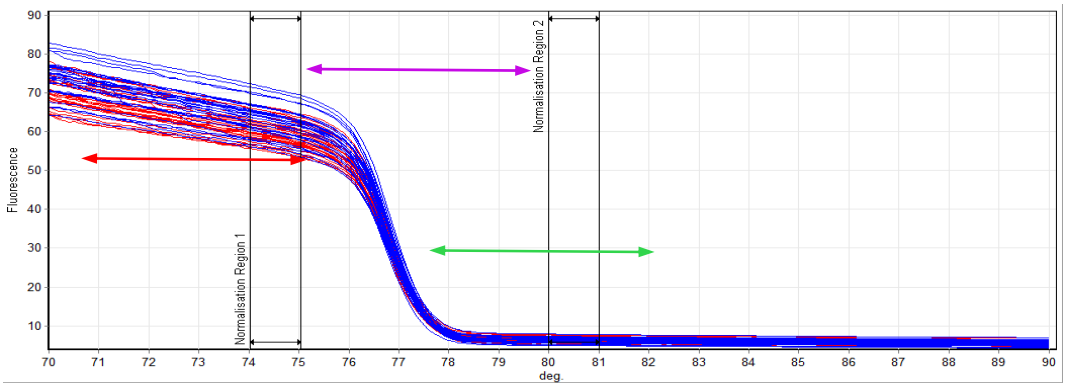
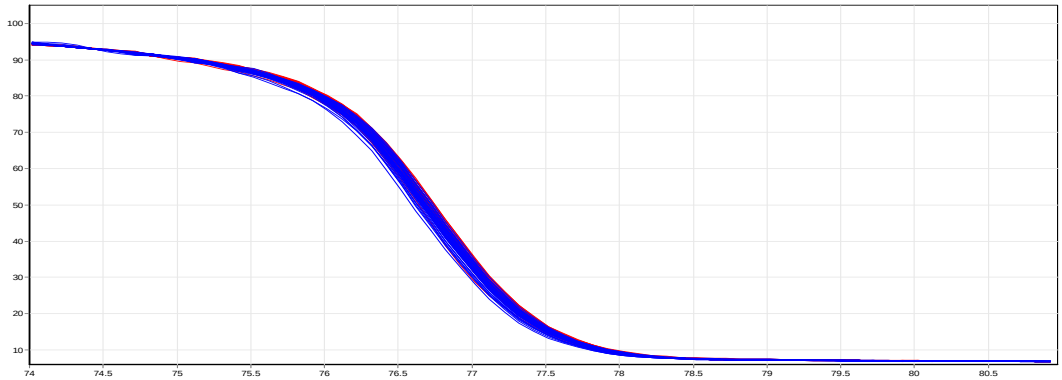


Figure 6.7 High resolution melt curve plots of raw data showing a range of initial fluorescence readings. Change of fluorescence for individual samples is difficult to discriminate in this view. Fluorescence is high at low temperature when the products are double-stranded (red-arrowed bar) and shift to low (green-arrowed bar) when it denatures and become single stranded. The active melt region (purple-arrowed bar) is designated by the pre-melt region (normalization region 1) and post melt (normalization region 2). These regions are utilized in aligning the data to provide a clearer view of the melt curve results (see Figure 6.8).

A. Normalized melt graph plots of IL21 gene fragment



B. Normalized melt graph plots of IL21R gene fragment

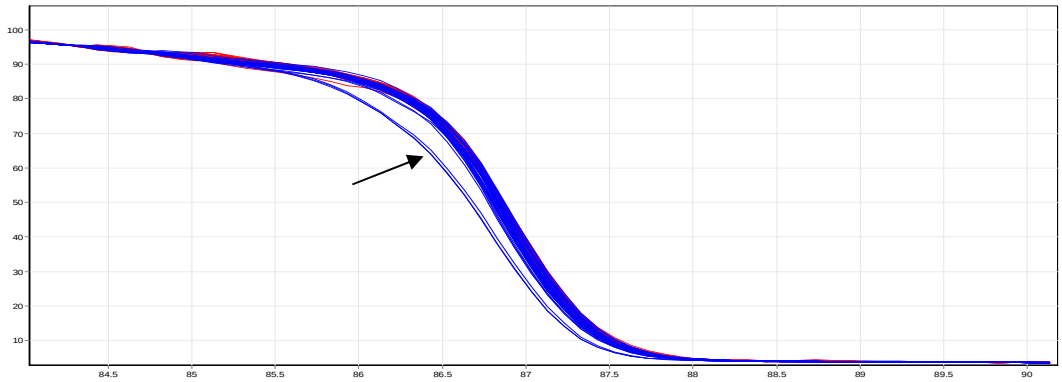
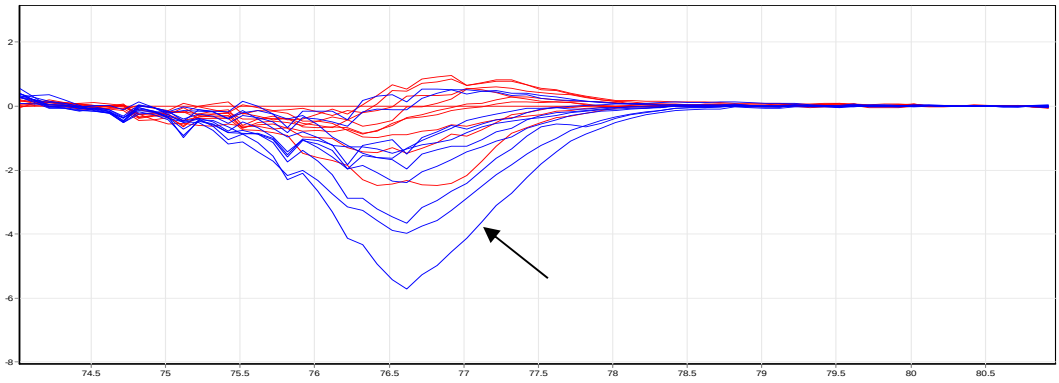


Figure 6.8 Normalized graphs (individual samples) in HRM analysis of resistant (red) and susceptible (blue) lambs. The x axis represents the normalized fluorescence and the y axis represents the temperature in degrees Celsius. This view is generated after setting the pre- and post-melt regions which provides a clear view of any temperature differences across samples. Temperature difference in samples pointed by arrow in a fragment of IL21R gene is evident (B) compared with a fragment of IL21 (A) where there is a fairly uniform melt temperature across samples.

A. Difference melt graph plots of IL21 gene fragment



B. Difference melt graph plots of IL21R gene fragment

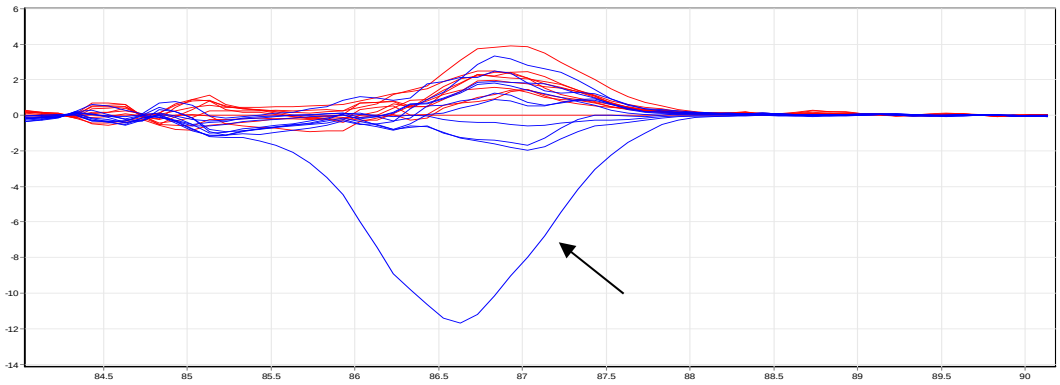


Figure 6.9 Difference melt curve plots of HRM analysis data (replicate view). The x axis represents the normalized fluorescence minus reference (control) and the y axis represents the temperature in degrees Celsius. Sample curves are subtracted from a reference curve (in this case, designated positive control sample). This view emphasizes differences between melt curves for each sample (arrows) which is very distinct in a fragment of IL21R gene (B) compared with a fragment of IL21 (A) where there is a fairly uniform melt cruve difference plots across samples.

6.2.8 SNP analysis

Analysis of the consensus IL21R gene fragment (158 bp) of sample 28 sequences revealed nucleotide base substitution from G to A at position 1261 (Figure 6.11A). Sequence containing potential SNP is shown aligned with the sequence of a sample (sample 65) with no detected difference in melt curve plots which contained no SNP. However, the nucleotide substitution did not result to any change in the translated protein (Figure 6.11B). No mutation was identified in IL21 gene fragment despite the slight difference in the melt curve plots observed.

The sample where SNP was identified (sample 28), and the reference sample which does not have SNP (sample 65) were both taken from the susceptible group. The sequences were analyzed based on the results of melt curve plots of HRM analysis (Figure 6.10).

(A)

```
CACAGTGACCGTGGTGGATGCAGAGGGGCTGTGTGACTGGCCCTGCACCTGTGGGGATGA  120

|||  ||||||||||||||||||||||||||||||||||||||||||||||||||||||||

CACGGTGACCGTGGTGGATGCAGAGGGGCTGTGTGACTGGCCCTGCACCTGTGGGGATGA  1317
```

(B)

```
                  *         20         *         40         *
sample_28 : LGSSVYSQEKDRLYGLVSIDTVTVVDAEGLCDWPCTCGDDGYPALNLDTGLE : 52
sample_65 : LGSSVYSQEKDRLYGLVSIDTVTVVDAEGLCDWPCTCGDDGYPALNLDTGLE : 52
            LGSSVYSQEKDRLYGLVSIDTVTVVDAEGLCDWPCTCGDDGYPALNLDTGLE
```

Figure 6.10 Nucleotide (A) and protein (B) sequence alignments of IL-21R fragment of sample with identified SNP. Position of SNP (G to A) at base 1261 of reference sequence 65 against sequence 28 is shown in the nucleotide sequence alignment.

6.3 Identification of *Ovar DRB*0203* allele

6.3.1 Extraction of genomic DNA from blood of 15 lambs

Genomic DNA was extracted (Section 2.8.1.5) from blood of 15 lambs representing 5 control and 5 each of the top resistant and top susceptible animals. The concentration of extracted DNA is presented in Table 6.5.

6.3.2 Detection of *Ovar DRB1*0203*

Amplification of MHC Class II Ovar *DRB1*0203* was performed in 15 lambs by using the forward primer 5'-TCTCTGCAGCACATTTCCTGG-3'(adopted from Schwaiger et al, 1995) and the reverse primer 5'-CACACACACACTGCTCCACA-3' (adopted from Sayers et al, 2005). This primer pair was employed to amplify the second exon of the *Ovar-DRB1* gene. The primers flank on the exon 2 region as the forward primer is located in intron 1 and the reverse primer in intron 3. Amplifications were performed for 30 cycles (1 min denaturation at 94ºC, 1 min annealing at 59 ºC, and 1 min extension at 72 ºC) in PCR Sprint cycler.

Table 6.6 DNA sample concentration used in *Ovar DRB*0203* study

Type	Sample	DNA concentration (ng/ul)	260/280 absorbance ratio
Control	1	27.71	1.84
	2	29.23	1.85
	3	21.96	1.75
	4	17.58	1.75
	5	32.83	1.89
Resistant	6	98.21	1.88
	7	85.42	1.91
	8	91.05	1.86
	9	34.67	1.77
	10	27.49	1.73
Susceptible	11	31.53	1.86
	12	26.61	1.91
	13	75.14	1.81
	14	33.43	1.91
	15	68.84	1.84

6.3.3 Identification of *Ovar DRB1*0203* allele in lambs that are resistant to *T. circumcincta*

PCR amplicons of *Ovar DRB1* in 15 lambs were obtained from genomic DNA. All PCR products were purified and sequenced as described in Section 1.2.4. A consensus sequence of *Ovar DRB1*0203* from three independent sequences for each sample was generated using CLC Sequence Viewer. It was not possible to generate the full nucleotide bases for the target amplicon in all but samples 9 and 13. This may possibly be due to mutationsthat could be present in the DNA of the samples or the presence of inhibitor in the DNA used. Sequence alignments, translations, and comparisons were carried out using ClustalW2. The BLAST algorithm was used to search the NCBI GenBank database for homologous sequences.

Figure 6.10 displays the alignments of nucleotide bases for each sample with the archetypal DRB1 and the published *Ovar DRB1*0203*. Alignment scores for these comparisons are shown Table 6.6. None of the 15 lambs across the three groups carry the *Ovar DRB*0203* allele. However, it is worth noting that the nucleic acid sequences of the resistant animals have close similarities with DRB*0203 which ranged from 83-98% compared to the susceptible group with similarities between 83-93%. This holds true in the translated sequences with 80-96% and 79-86% matched amino acid in resistant and susceptible animals respectively.

```
Ovar-DRB01      --AGTATACTAAGAAAGAGTGTCGTTTCTCCAACGGGACGGAGCGGGTGCGGTTCCTGGA 58
Ovar-DRB1*0203  GGAGTATTCTACGAGCGAGTGTCATTTCTTCAACGGGACGGAGCGGGTGCGGTTCCTGGA 60
Ovar-DRB1_1     ---------------------------------CGGGACGGAGCGGGTGCGGTTCCTGGA 27
Ovar-DRB1_2     ------------------------TTCTCCAACGGGACGGAGCGGGTGCGGTTCCTGGA 35
Ovar-DRB1_3     --------------------------------CGGGACGGAGCGGGTGCGGTTCCTGGA 27
Ovar-DRB1_4     -------------------------------ACGGGACGGAGCGGGTGCGGTTCCTGGA 28
Ovar-DRB1_5     ----------------------------------GGACGGAGCGGGTGCGGTTCCTGGA 25
Ovar-DRB1_6     --------------------------------CGGGACGGAGCGGGTGCGGTTCCTGGA 27
Ovar-DRB1_7     --------------------------------CGGGACGGAGCGGGTGCGGTTCCTGGA 27
Ovar-DRB1_8     -------------------------TCTCCAACGGGACGGAGCGGGTGCGGTTCCTGGA 34
Ovar-DRB1_9     --AGTATTCTACGAGCGAGTGTCATTTCTTCAACGGGACGGAGCGGGTGCGGTTCCTGGA 58
Ovar-DRB1_10    --------------------------------CGGGACGGAGCGGGTGCGGTTCCTGGA 27
Ovar-DRB1_11    --------------------------------CGGGACGGAGCGGGTGCGGTTCCTGGA 27
Ovar-DRB1_12    --------------------------------CGGGACGGAGCGGGTGCGGTTCCTGGA 27
Ovar-DRB1_13    --AGTATACTAAGAAAGAGTGTCGTTTCTCCAACGGGACGGAGCGGGTGCGGTTCCTGGA 58
Ovar-DRB1_14    ---------------------------------GGGACGGAGCGGGTGCGGTTCCTGGA 26
Ovar-DRB1_15    -------------------------CTCCAACGGGACGGAGCGGGTGCGGTTCCTGGA 33
                                         ************************

Ovar-DRB01      CAGATACTTCCATAATGGAGAAGAGACCCTGCGCTTCGACAGCGACTGGGGCGAGTACCG 118
Ovar-DRB1*0203  CAGATACTTCTATAATGGAGAAGAGACCCTGCGCTTCGACAGCGACTGGGGCGAGTACCG 120
Ovar-DRB1_1     AAGATACTTCTATAATGGAGAAGAGACCCTGCGCTTCGACAGCGACTGGGGCGAGTACCG 87
Ovar-DRB1_2     AAGATACTTCTATAATGGAGAAGAGACCCTGCGCTTCGACAGCGACTGGGGCGAGTACCG 95
Ovar-DRB1_3     CAGATACTTCTATAATGGAGAAGAGACCCTGCGCTTCGACAGCGACTGGGGCGAGTACCG 87
Ovar-DRB1_4     CAGATACTTCCATAATGGAGAAGAGACCCTGCGCTTCGACAGCGACTGGGGCGAGTACCG 88
Ovar-DRB1_5     CAGATACTTCCATAATGGAGAAGAGACCCTGCGCTTCGACAGCGACTGGGGCGAGTACCG 85
Ovar-DRB1_6     CAGATACTTCCATAATGGAGAAGAGACCCTGCGCTTCGACAGCGACTGGGGCGAGTACCG 87
Ovar-DRB1_7     CAGATACTTCCATAATGGAGAAGAGACCCTGCGCTTCGACAGCGACTGGGGCGAGTACCG 87
Ovar-DRB1_8     CAGATACTTCTATAATGGAGAAGAGACCCTGCGCTTCGACAGCGACTGGGGCGAGTACCG 94
Ovar-DRB1_9     CAGATACTTCTATAATGGAGAAGAGACCCTGCGCTTCGACAGCGACTGGGGCGAGTACCG 118
Ovar-DRB1_10    CAGATACTTCCATAATGGAGAAGAGACCCTGCGCTTCGACAGCGACTGGGGCGAGTACCG 87
Ovar-DRB1_11    NAGATACTTCTATAATGGAGAAGAGACCCTGCGCTTCGACAGCGACTGGGGCGAGTACCG 87
Ovar-DRB1_12    CAGATACTTCTATAATGGAGAAGAGACCCTGCGCTTCGACAGCGACTGGGGCGAGTACCG 87
Ovar-DRB1_13    CAGATACTTCCATAATGGAGAAGAGACCCTGCGCTTCGACAGCGACTGGGGCGAGTACCG 118
Ovar-DRB1_14    CAGATACTTCCATAATGGAGAAGAGACCCTGCGCTTCGACAGCGACTGGGGCGAGTACCG 86
Ovar-DRB1_15    CAGATACTTCCATAATGGAGAAGAGACCCTGCGCTTCGACAGCGACTGGGGCGAGTACCG 93
                 ********* ************************************************

Ovar-DRB01      AGCGGTGGCCGAGCTGGGGCGGCCGGACGCTAAGTACTGGAACAGCCAGAAGGACTTCCT 178
Ovar-DRB1*0203  AGCGGTGGCCGAGCTGGGGCGGCCGGACGCCAAGTACTGGAACAGCCAGAAGGAGATCCT 180
Ovar-DRB1_1     AGCGGTGGCCGAGCTGGGGCGGCCGGACGCCAAGTACTGGAACAGCCAGAAGGAGCTCCT 147
Ovar-DRB1_2     AGCGGTGGCCGAGCTGGGGCGGCCGGACGCCAAGTACTGGAACAGCCAGAAGGAGCTCCT 155
Ovar-DRB1_3     AGCGGTGGCCGAGCTGGGGCGGCCGGACGCCAAGTACTGGAACAGCCAGAAGGACCTCCT 147
Ovar-DRB1_4     AGCGGTGGCCGAGCTGGGGCGGCCGGACGCTAAGTACTGGAACAGCCAGAAGGACTTCCT 148
Ovar-DRB1_5     AGCGGTGGCCGAGCTGGGGCGGCCGGACGCCAAGTACTGGAACAGCCAGAAGGACTTCCT 145
Ovar-DRB1_6     AGCGGTGGCCGAGCTGGGGCGGCCGGACGCCAAGTACTGGAACAGCCAGAAGGACTTCCT 147
Ovar-DRB1_7     AGCGGTGGCCGAGCTGGGGCGGCCGGACGCCAAGTACTGGAACAGCCAGAAGGACTTCCT 147
Ovar-DRB1_8     AGCGGTGGCCGAGCTGGGGCGGCCGGACGCCAAGTACTGGAACAGCCAGAAGGACNTCCT 154
Ovar-DRB1_9     AGCGGTGGCCGAGCTGGGGCGGCCGGACGCCAAGTACTGGAACAGCCAGAAGGAGNTCCT 178
Ovar-DRB1_10    AGCGGTGGCCGAGCTGGGGCGGCCGGACGCCAAGTACTGGAACAGCCAGAAGGACTTCCT 147
Ovar-DRB1_11    AGCGGTGGCCGAGCTGGGGCGGCCGGACGCCAAGTACTGGAACAGCCAGAAGGACTTCCT 147
Ovar-DRB1_12    AGCGGTGGCCGAGCTGGGGCGGCCGGACGCCAAGTACTGGAACAGCCAGAAGGACTTCCT 147
Ovar-DRB1_13    AGCGGTGGCCGAGCTGGGGCGGCCGGACGCTAAGTACTGGAACAGCCAGAAGGACTTCCT 178
Ovar-DRB1_14    AGCGGTGGCCGAGCTGGGGCGGCCGGACGCCAAGTACTGGAACAGCCAGAAGGACTTCCT 146
Ovar-DRB1_15    AGCGGTGGCCGAGCTGGGGCGGCCGGACGCCAAGTACTGGAACAGCCAGAAGGACTTCCT 153
                ***************************** ********************  ****

Ovar-DRB01      GGAGCGGGCGCGGGCCGCCGTGGACACGTACTGCAGACACAACTACGGGGTCATTGAGAG 238
Ovar-DRB1*0203  GGAGCGGAAGCGGGCCGCCGTGGACACGTACTGCAGACACAACTACGGGGTCGGTGA--- 237
Ovar-DRB1_1     GGAGCGGAAGCGGGCCAATGTGGACACGTACTGCAGACACAACTACGGGGTCGGTGAGAG 207
Ovar-DRB1_2     GGAGCGGAAGCGGGCCAATGTGGACACGTACTGCAGACACAACTACGGGGTCGGTGAGAG 215
Ovar-DRB1_3     GGAGCGGAAGCGGGCCAACGTGGACACGTACTGCAGACACAACTACGGGGTCATTGAGAG 207
Ovar-DRB1_4     GGAGCGGANGCGGGCCGCCGTGNACACGTACTGCAGACACAACTACGGGGTCTTTGAGAG 208
Ovar-DRB1_5     GGAGCGGACGCGGACCGCCGTGGACACGTACTGCAGACACAACTACGGGGTCATTGAGAG 205
Ovar-DRB1_6     GGAGCGGACGCGGGCCGCCGTGGACACGTACTGCAGACACAACTACGGGGTCATTGAGAG 207
Ovar-DRB1_7     GGAGCGGAAGCGGGCCGCCGTGGACACGTACTGCAGACACAACTACGGGGTCATTGAGAG 207
Ovar-DRB1_8     GGAGCGGAAGCGGGCCGCCGTGGACACGTACTGCAGACACAACTACGGGGTCATTGAGAG 214
Ovar-DRB1_9     GGAGCGGAAGCGGGCCGCCGTGGACACGTACTGCAGACACAACTACGGGGTCNGTGAGAG 238
Ovar-DRB1_10    GGAGCGGGCGCGGGCCGCCGTGGACACGTACTGCAGACACAACTACGGGGTCATTGAGAG 207
Ovar-DRB1_11    GGAGCGGACGCGGGCCGCCGTGGACACGTACTGCAGACACAACTACGGGGTCATTGAGAG 207
Ovar-DRB1_12    GGAGCGGACGCGGGCCGCCGTGGACACGTACTGCAGACACAACTACGGGGTCATTGAGAG 207
Ovar-DRB1_13    GGAGCGGGCGCGGGCCGCCGTGGACACGTACTGCAGACACAACTACGGGGTCATTGAGAG 238
Ovar-DRB1_14    GGAGCGGACGCGGGCCGCCGTGGACACGTACTGCAGACACAACTACGGGGTCATTGAGAG 206
Ovar-DRB1_15    GGAGCGGAAGCGGGCCGCCGTGGACACGTACTGCAGACACAACTACGGGGTCATTGAGAG 213
                *******. ****.**.. ***.***************************  ***
```

Figure 6.11 Sequence alignments of Ovar-DRB1 allele in 15 samples: 1 to 5 (Control), 6 to 10 (Resistant), and 11 to 15 (Susceptible) together with the published archetypal Ovar-DRB1 (Accession no. U00204.1) and *Ovar-DRB*0203* (AB017206.1). (*) indicates alignment with *Ovar-DRB1*.

Table 6.7 Identity level of nucleic acid and amino acid sequences of the MHC Class II *Ovar DRB1* genes of 15 lambs with reference to published sequence of *Ovar-DRB1*0203*.

Sample		Alignment scores (%)	
		Nucleic acid	Amino acid
Control	1	83	78
	2	86	84
	3	90	86
	4	83	78
	5	89	83
Resistant	6	87	82
	7	83	80
	8	86	94
	9	98	96
	10	88	83
Susceptible	11	83	79
	12	90	86
	13	93	83
	14	87	82
	15	86	82

```
Ovar-DRB1           GTERVRFLDRYFHNGEETLRFDSDWGEYRAVAELGRPDAKYWNSQKDFL 59
Ovar-DRB1*0203      ------------Y--------------------------------EI- 60
Ovar-DRB1_1         ------------------------------------------------ 49
Ovar-DRB1_2         --------E-----------------------------------YEL- 52
Ovar-DRB1_3         ------------Y-----------------------------------L- 49
Ovar-DRB1_4         ------------------------------------------------ 49
Ovar-DRB1_5         ------------------------------------------------ 48
Ovar-DRB1_6         ------------------------------------------------ 49
Ovar-DRB1_7         ------------------------------------------------ 49
Ovar-DRB1_8         ------------------------------------------------ 51
Ovar-DRB1_9         ----------------------------------------------EX- 59
Ovar-DRB1_10        ------------------------------------------------ 49
Ovar-DRB1_11        --------X---Y----------------------------------- 49
Ovar-DRB1_12        ------------Y----------------------------------- 49
Ovar-DRB1_13        ------------------------------------------------ 52
Ovar-DRB1_14        ------------------------------------------------ 49
Ovar-DRB1_15        ------------------------------------------------ 51

Ovar-DRB1      ERARAAVDTYCRHNYGVIESFTVQRRGERGGGRPLWSSVCVCVCVCVCVCVCVCVERE 119
Ovar-DRB1*0203 --K--------------G---------------------------------------- 78
Ovar-DRB1_1    --X----X---------F--------------L--------------------------- 90
Ovar-DRB1_2    --K—N------------G---------------------------------------- 90
Ovar-DRB1_3    --K—N------------------------------------------------------ 73
Ovar-DRB1_4    --X----X---------F--------------L--------------------------- 90
Ovar-DRB1_5    --T-T------------------------------------------------------ 72
Ovar-DRB1_6    --T----------------------R---------------------------------- 75
Ovar-DRB1_7    --K------------------------------------------------------- 90
Ovar-DRB1_8    --K------------------------------------------------------- 69
Ovar-DRB1_9    --K--------------X---------------X------------------------ 97
Ovar-DRB1_10   --A------------------------------------------------------- 74
Ovar-DRB1_11   --T------------------------------------------------------- 78
Ovar-DRB1_12   ------------------------------------------------------------ 73
Ovar-DRB1_13   ------------------------------------------------------------ 78
Ovar-DRB1_14   --T------------------------------------------------------- 75
Ovar-DRB1_15   --K------------------------------------------------------- 81
```

Figure 6.12 Protein translation of the second exon of *Ovar-DRB1* allele in 15 samples: 1 to 5 (Control), 6 to 10 (Resistant), and 11 to 15 (Susceptible). Each protein translation is relative to the archetypal Ovar-DRB1 (Accession no. U00204.1). Translation of allele *Ovar-DRB1*0203* which has been linked to resistance to *T. circumcincta* infection is also shown. Dash indicates identity in the amino acid sequence to the sequence of *Ovar DRB1*.

6.4 Conclusion

The full length 1,596 base sequence of the coding region for ovine IL21R was generated. A partial sequence for the ovine IL21 transcript was produced. Their genomic organization as to the position of exons and introns was described in relation to the ovine genome. An optimized assay for high resolution melt analysis in detecting SNPs in ovine IL21 and IL21R was developed. A fragment (158 bp long) of IL21R coding region showed one nucleotide base substitution from G to A in one sheep, with no alteration in the translated protein.

The allele MHC Class II *Ovar DRB*0203* was not identified in the population of lambs used in the study. Therefore, we could not link the resistance of the lambs to *T. circumcincta* with the expression of this gene.

Chapter 7 Discussion

7.1 Overview

To examine the immunology of parasite resistance in sheep, lambs with diversity in their predicted genetic resistance to *T. circumcincta* were exploited to investigate the association between the T cell response and phenotypic parameters of parasite infection. Naïve lambs were trickle-infected three times per week over a period of three months to simulate a chronic natural infection, which resulted in animals with range of susceptibilities that reflected the nature and magnitude of the mature immune response. Expression of protective immunity in the maturation of the acquired immune response is thought to have commenced after about 50 days of continuous infection as FEC was high in all animals until after this time point (Beraldi *et al.*, 2008). At post mortem, after 13 weeks of continual infection, adult worms were highly aggregated in few lambs and absent or low in number in resistant lambs. It is interesting that a population of lambs exposed to similar level of infection under identical management systems resulted in clearance of infection in some and persistence of infection in others.

Data pertaining to the histopathology and immunological response were gathered and analyzed during the stage when the mature immune response of the resistant animals was controlling and/or eliminating recurrent parasite infection. It was also during this stage that other animals in the group did not exhibit control of the infection thereby retaining mature adult nematodes and excreting numbers of parasite eggs (Beraldi *et al.*, 2008).

I hypothesized that resistance/susceptibility to *T. circumcincta* is associated with differential activation of Treg and TH17 T cells as well as the interaction between

TH1 and TH2 subsets. These four CD4+ T cell subsets were investigated by measurement of the transcripts of their characteristic markers and effector cytokines and quantitative expression was correlated with individual traits of resistance and susceptibility. Genes that are associated with the immune response linked to resistance or susceptibility may be candidates that could be developed as markers for selective breeding. Attempts were made to identify mutations in differentially-expressed genes of susceptibility to *T. circumcincta* infection.

7.2 Development of assay

Analysis of the immune response required the measurement of ovine transcripts particularly for those that were not available from published sequence databases. Protein expression as well as functional and immunological assays like FACS, immunohistochemistry and western blots are ideal and desirable but are insensitive for cytokines, especially within tissues. Furthermore, these assays are limited by lack of antibodies available for sheep and by not being inherently quantitative.

In order to evaluate the expression of genes associated with *T. circumcincta* infection, I developed real-time RT-qPCR assays for lowly-expressed ovine transcripts. Bovine templates were utilized initially to design primers for these genes using stringent criteria (Untergasser *et al.*, 2007) to ensure that only the genes of interest were amplified. Analysis of the generated ovine sequences has verified its similarity of at least 95% to closely-related bovine species for all genes included in the study. This gave me more confidence that measurements were made specifically on the target transcripts. This step also conforms to the 'Minimum information for

publication of quantitative real–time experiments (MIQE) guidelines to ensure the reliability of assay results (Bustin, 2010; Bustin *et al.*, 2009; Taylor *et al.*, 2010).

It has been emphasized how primer and magnesium concentration as well as annealing temperature affects the melting temperature specificity and overall efficiency of the real-time RT-qPCR assay (Eckert and Kunkel, 1991; Mikeska and Dobrovic, 2009; Rychlik *et al.*, 1990). Primer and magnesium optimization steps were found to be critical as shortage or an excess of what is optimum results to non-specific product formation. The method instituted to determine the optimal primer and magnesium concentration for real-time RT-qPCR is in agreement with previous assay validation experiments (Bustin, 2004; Bustin *et al.*, 2011; Mikeska and Dobrovic, 2009).

All the parameters mentioned above were optimized, and throughout the conduct of the assay various sources of errors due to poor technical skills and slack laboratory practice were encountered. These include minor but equally important steps like pipetting errors, reagent preparation and storage, and risks of contamination. Problems were addressed by the use of automated liquid handling system as described previously (Section 2.5.1) and making aliquots for reagents that require frequent freeze-and-thaw. Use of filter tips and gloves, applying decontaminating agents on workbench before setting up experiments, and setting a dedicated room for amplified products away from the PCR set-up area reduced contamination. This was done to ensure that only the parameter in question was analyzed in each RT-qPCR run. All RT-qPCR reactions had an efficiency of >90% and r^2 of >0.98. These assessment criteria, including melt curve analysis and gel electrophoresis, conform to

the requisites of a workable real-time RT-qPCR assay according to MIQE guidelines (Bustin *et al.*, 2009). The developed assay can now be utilized in related studies defining biological systems, particularly disease processes in sheep.

7.3 Inappropriate inflammatory response do not clear worm infection

The histopathology of resistant and susceptible lambs reflected the differential immune response to larval challenge with *T. circumcincta*. The pathological consequence of *T. circumcincta* infection consists of tissue injury and local cellular changes, which may be to the result of host immune response (McKellar, 1993; Stear *et al.*, 2003). In this study, the extent of tissue damage based on epithelial cell hyperplasia could not be determined because tissue blocks were taken from different regions of the abomasum where baseline counts differ. Likewise, the inflammatory infiltrates were not quantified by systematic scoring system used in characterizing the degree of pathology (Scott *et al.*, 1998). The cell counts which ranged from zero to <10 was deemed too low to draw a quantitative cellular profile (Beard, 2010). Mast cell counts could have been useful as these cells have been shown to be important in the inhibition of larval establishment (Huntley *et al.*, 1992). However, this could not be possible as the fixative used (zinc salt) was found to be incompatible with efficient toluidine blue staining.

Despite the abovementioned limitations, the histopathological profile of resistant and susceptible animals was clearly differentiated. The abomasal mucosa of resistant lambs showed only minor pathological changes, with the gastric glands empty of larvae despite uninterrupted, three times weekly infection with ~2000 infective larvae. Resistant lambs with zero to low worm load showed mild inflammation with

neutrophils and eosinophils only seen occasionally. In contrast, susceptible lambs showed extensive lymphocytic infiltration characteristic of a chronic inflammatory response (Salman and Duncan, 1984). Although nematodes were not seen in the gastric mucosa, the vacuolated areas surrounded by neutrophils, eosinophils and cellular debris indicate that larval migration has taken place.

The mild pathology in resistant animals suggests strongly that the L3 larvae may have failed to establish to the adult stage and/or the mature adults may have been expelled. The cellular profile of the abomasal tissue in resistant animals consisting of mild lymphocytic infiltrate with few eosinophils and neutrophils may be consequent to scantiness of colonizing worms hence, the low level of pathology (Smith *et al.*, 1984; Stear *et al.*, 1999a). Likewise, it seems reasonable to assume that susceptible lambs were unable to control parasite colonization/establishment and egg production (Miller, 1996; Miller, 1984).

Worm clearance in resistant animals with little pathology is consistent with the generalization that acquired immunity to GI nematodes can be achieved after weeks of continuous low-level exposure (Gamble and Zajac, 1992; Seaton *et al.*, 1989; Vanimisetti *et al.*, 2004). On the other hand, persistence of infection with consequent tissue damage may be attributed to ineffective or incomplete immunity (Meeusen *et al.*, 1995; Meeusen, 1999).

The present findings suggest that an active immune response is elicited in chronic *T. circumcincta* infection but the inflammatory response for susceptible animals is inappropriate to effectively clear the infection.

7.4 TH17-related cytokines are up-regulated in susceptible lambs

It is clear from several studies of mouse models of gastrointestinal nematode infection that the type of T helper response is critical to the outcome of infection (Else and Finkelman, 1998; Gause *et al.*, 2003; Grencis, 2001). Protective immunity ensues in a predominant TH2 response where cytokines orchestrate effector mechanisms to eliminate infection. On the contrary, protection is compromised where TH1 response gains hierarchy. The cytokines produced in TH2 responses in the relative absence of TH1 promote increased number of mast cells, eosinophils and elevated levels of IgE and IgG1 in mice (Anthony *et al.*, 2007; Gause *et al.*, 2003). Mastocytosis and eosinophilia are also observed in sheep -in addition to the high expression of IgA antibody (Harrison *et al.*, 2003; Strain *et al.*, 2002). IgA activity is thought to be the main immunological mechanism regulating worm length and fecundity which translates to reduced gastrointestinal worm infection (Stear *et al.*, 1999a) in sheep.

In this study, fold change analysis revealed increased expression of IL6 and IL21 in the abomasal lymph node as well as IL6 and TGFB1 in the abomasal mucosa of the susceptible group of lambs in comparison to resistant animals. Results of transcript copy number measurements in the abomasal lymph node of the full cohort of 55 animals were consistent with the fold change data on the elevated expression of IL6 and IL21 in susceptible lambs. IL23A expression was also significantly increased in the susceptible group.

The cytokine profile in these analyses is consistent with the hypothesis that susceptibility to *T. circumcincta* infection is related to the orchestration of an

inflammatory TH17 response (Harrington *et al.*, 2006; Weaver *et al.*, 2007). Susceptible animals had the highest level of IL6, IL21 and IL23A transcripts in the abomasal lymph node while retaining consistent levels of TGFB1. The characteristic up-regulation of these cytokines is reminiscent of the chronic inflammatory response associated with autoimmune inflammatory diseases (Izcue *et al.*, 2009; Kaser *et al.*, 2010). Indeed, high levels of IL6 and IL23 were found in untreated inflamed mucosa of patients with inflammatory bowel disease (Olsen *et al.*, 2011). Moreover, an over-expression of IL21 in inflamed intestine of IBD patients has been reported (Monteleone *et al.*, 2009). These cytokines are primarily involved in the initiation and maintenance of the TH17 response and they have all been shown to induce STAT3 phosphorylation (Boniface *et al.*, 2008). The pathology associated with the inflammatory response in the abomasum of susceptible lambs may be analogous to CD and UC which are two of the most common forms of autoimmune IBD (Weaver *et al.*, 2006).

IL6 expression was consistently high in lymph node and abomasum of susceptible animals. This pro-inflammatory cytokine drives the TGFβ-induced T cells to differentiate to TH17 cells in mice (Mangan *et al.*, 2006; Veldhoen *et al.*, 2006). Stabilization of the TH17 response is attributed to IL-23 expression relying on IL-23R which is not expressed in naïve T cells (Parham *et al.*, 2002). IL-6 enhances the activation of IL-23R and works synergistically with IL-23 in further promoting the TH17 response (Morishima *et al.*, 2009).

IL21 has the highest expression in susceptible animals among all transcripts measured. Sustaining the TH17 response is thought to be mediated by IL-21 (Yang

et al., 2008b) that amplifies the production of TH17 cells, which in turn selectively produce IL-21 (Peluso *et al.*, 2007). The autocrine production of IL-21 intensifies the availability of IL21, which has several downstream functions that maintains the TH17 response. IL-21 not only activates STAT3 (Brenne *et al.*, 2002) but also triggers the expression of RORγt (Fantini *et al.*, 2007); both important in the lineage commitment to the TH17 response. Furthermore, IL-21 induces the production of the key cytokines IL-17A and IL-17F that characterizes the TH17 response (Mills, 2008).

Further expansion of TH17 from naïve T cells also seems to be controlled by IL-23 released by dendritic cells and macrophages (Collins *et al.*, 2011; Kastelein *et al.*, 2007) (Kastelein *et al*., 2007; Ahern *et al*., 2010). This cytokine has been shown to be critical for the stability and pathogenicity of TH17 cells (Lexberg *et al.*, 2008; McGeachy *et al.*, 2007). IL-23 is an IL-12-related key inflammatory cytokine (Duvallet *et al.*, 2011; Iwakura and Ishigame, 2006; Tang *et al.*, 2011) that is involved in the establishment of chronic inflammation and in the development of TH17 cells. The elevated levels of IL-23 in susceptible lambs may be related to the massive inflammatory response in the abomasum of these lambs that are carrying a high parasite load.

The participation of IL-23 in the inflammatory response may be attributed to the proliferation of TH1 cells as it has heterodimer link with IL-12. However, its co-expression with other TH17 cytokines, like IL-6 and IL-21, in susceptible lambs suggests that the pathway led to TH17 response. Recent studies, based on the presence of p19 and p40 subunits of IL-23 and not IL-12 p35, have indicated that IL-

23 is the dominant cytokine controlling inflammation in peripheral tissues (Goldberg *et al.*, 2009; Lee *et al.*, 2004). Evidence shows that IL-23 may induce an autocrine loop within the innate immune system leading to production of numerous mediators of inflammation (Yen *et al.*, 2006). The observed inflammatory response is consistent with the pathology of IBD (Liu *et al.*, 2011; Olsen *et al.*, 2011) in human patients. *In vitro* studies show that IL-17A expression is dependent on continued expression of IL-23 and a combination of TGFβ/IL6 in mice and TGFβ/IL-21 in humans (Lexberg *et al.*, 2008).

An argument against the ascendancy of the TH17 response in susceptibility to *T. circumcincta* infection is that IL-17A could not be detected in any sheep in this experiment. IL-17A is the signature TH17 cytokine and was not present in detectable amount in tissues examined in this study, which may be due to the fact that this cytokine is only produced early after activation and diminishes within a few days (Lohr *et al.*, 2009). It must be noted however that the sheep tissues were from persistently infected animals undergoing mature immune responses and had been continuously stimulated for 13 weeks. Tissues were collected 2-3 days after the last larval challenge and it is probable that subsequent re-infection failed to induce IL-17A production, or that it was activated but could only persist for less than 3 days.

The involvement of inflammatory CD4+ T cells in susceptibility of lambs to *T. circumcincta* is evidenced by the recent digital gene expression experiments showing significantly higher expression of H2.0-like homeobox (HLX) and TGFBR1 transcripts in susceptible lambs (Pemberton *et al.*, 2011). HLX is a known negative regulator of IFNγ through the targeted depletion of STAT4, a key transcription factor

for IFNγ mRNA synthesis in NK cells (Becknell *et al.*, 2007) and may signal a positive feedback for other TH cells, for example TH17 cells to proliferate. However, in mature TH1 cells HLX is induced by T-bet leading to optimum production of IFNγ (Mullen *et al.*, 2002).

TGFβ receptor1 works with TGFβ which is involved in the differentiation of TH17 and Treg cells. Expression of TGFβ receptor has been associated with inflammatory autoimmune disorders (Shimizu *et al.*, 2012). Additionally, a global transcriptome analysis of the ovine immune response to acute *T. circumcincta* infection revealed high expression of transcripts like heat shock proteins, complement factors, and chemokines that are involved in direct parasitic effects, immunomodulation, and tissue repair (Knight *et al.*, 2011).

7.5 TH17 versus Treg proliferation

There are indications that Treg and TH17 cells are not a stable phenotype; mature Tregs can re-express RORγt and can be converted to IL-17 producing cells (Ariana and Elizabeth, 2009; Yang *et al.*, 2008c).

IL-21 or IL-6 may act as a switch leading to TH17 or Treg differentiation. Activation of Treg development from naïve CD4+ T cells occurs in the presence of TGFβ, however expression of the same cytokine in combination with IL-21 (Mucida *et al.*, 2007; Yang *et al.*, 2008b) or IL-6 (Kimura *et al.*, 2007) directs the development of TH17 cells. Both cytokines prevent the TGFβ-dependent expression of FOXP3 promoting the differentiation of TH17 instead of Treg cells (Bettelli *et al.*, 2006; Fantini *et al.*, 2007; Fujimoto *et al.*, 2011; Veldhoen *et al.*, 2006). High

expression of IL6 and IL21 in susceptible lambs may have triggered the shift to TH17 from Treg cells.

Previous studies suggest that expansion of TH17 and Treg subsets is more dependent on TGFβ (Bettelli *et al.*, 2006; Mangan *et al.*, 2006; Veldhoen *et al.*, 2006) than IL-6. In my study, TGFB1 was elevated in the abomasal mucosa of susceptible lambs compared to the other groups. This cytokine alone could have favoured differentiation of Treg cells with consequent down-regulation of TH17 cells (Ivanov *et al.*, 2007; Lee *et al.*, 2009). However, increased IL6 levels may have favourably driven the expansion of TH17 cells. This corroborates studies in mouse models showing that the combination of IL-6 and TGFβ is essential to drive TH17 differentiation (Bettelli *et al.*, 2006; Veldhoen and Stockinger, 2006). However, the participation of TGFβ in TH17 cell differentiation is not clear. Although it was demonstrated that neutralizing antibody against TGFβ almost completely blocked IL-6 and IL-23 (Morishima *et al.*, 2009) it has also been shown that TGFβ can be dispensable in the orchestration of murine TH17 cell development (Ghoreschi *et al.*, 2011). Additionally, it is probable that the measured TGFB1 transcripts do not represent the biologically active, mature TGFβ (Barcellos-Hoff, 1996). TGFβ can persist in extracellular components as latent complex that requires stimulation by proteolytic, conformational and other factors to be activated (Abe *et al.*, 2002; Barcellos-Hoff, 1996; Munger *et al.*, 1999). Thus, whether TGFβ promotes TH17 differentiation directly by up-regulating its expression or indirectly by down-regulating the expression of cytokines related with other TH cell subtypes is not clear.

The elevated levels of FOXP3 in both infected resistant and susceptible lambs compared to uninfected control animals may indicate that immune-regulation is at play. TH17 and Treg cell subsets are both known to play a significant role in mucosal immunity by protecting the epithelial cells from invading pathogens (Bettelli *et al.*, 2006; Harrington *et al.*, 2005). The reciprocal relationship of Treg and TH17 cells may be reflected in the expression of RORγt being inhibited, as induced Treg cell differentiation progresses (Zhou *et al.*, 2008).

Like in other chronic inflammatory diseases (Olsen *et al.*, 2011; Wong *et al.*, 2001), either or both IL-6 and IL-21 may have contributed in the differentiation ofTH17 cells in susceptible lambs with chronic *T. circumcincta* infection. IL-21 may direct the expansion of TH17 cells instead of Treg cells by preventing the TGFβ-dependent expression of FOXP3 (Fantini *et al.*, 2007). Consequently, autocrine secretion of IL-21 is activated which further enhances the TH17 response. The same mechanism applies to IL-6 which blocks FOXP3 thereby preventing Treg differentiation and favouring TH17 response (Bettelli *et al.*, 2006; Fantini *et al.*, 2007).

7.6 What tips the balance to TH cell phenotype?

As I was only able to demonstrate the profile of the immune response at the resolution stage of *T. circumcincta* infection in resistant animals, the series of events that took place before this stage can be presumed based on previous related work. TH1 may have been orchestrated at the initiation of worm infection (Pernthaner *et al.*, 2006) which may have been followed eventually by the development of protection, characterized by TH2 response (Andronicos *et al.*, 2010) with continued worm challenge. This effector response may lead to pathology if uncontrolled,

prompting Treg cells to suppress the proliferation of TH1 and TH2 cells. This could be the default response in chronic *T. circumcincta* infection. However, the results of this study suggest that in selected susceptible lambs, the cytokine milieu at the maturation of the immune response can inhibit Treg cell development and function, in favour of the inflammatory TH17 response. This concurs with the reported adoption of TH17-like phenotype by human and murine Tregs upon activation in an inflammatory cytokine milieu (Ayyoub *et al.*, 2012; Radhakrishnan *et al.*, 2008).

As with other gastrointestinal helminth parasites, protective immunity to *T. circumcincta* seems to be associated with the conventional TH2 response (Craig *et al.*, 2007; Finkelman *et al.*, 1991; Lacroux *et al.*, 2006). However, in the experiments reported here, expression levels of transcripts of the classic type 1 cytokines IL-12 and IFNγ and the type 2 cytokines IL-4, IL-25 and IL-10, did not correlate with susceptibility and resistance respectively. Very few IL4 and IL25 transcripts were detected in any animal; while IL10 and IL12B showed no significant changes and IFNG was significantly reduced in susceptible animals. These findings are contrary to previous reports on up-regulated expression of IL-4 and IL-10 in TH2-mediated protection to GI nematodes in sheep during acute infections (Hein *et al.*, 2004; Lacroux *et al.*, 2006; Meeusen *et al.*, 2005). The low expression of these cytokines may be explained by TH2 cells proliferating during acute nematode infection, leading to the development of protective IgA antibody. This antibody leads to the expulsion of adult worms and inhibits new L3 larval colonization thereby obviating continual activation (Pearce *et al.*, 2004; Taylor *et al.*, 2009). Negligible levels of IL4 and IL25 in all study animals and down-regulated IFNG and EBI3

expression in susceptible lambs may have also arisen from immune-suppression by Treg cells as this were concurrent with high FOXP3 expression.

Regulation of TH17 cells is consistent with recent observations on blood fluke *S. mansoni* infection in mice, suggesting that lower levels of the TH17-related cytokine IL-17A would result in the generation of protective responses associated with IgG antibody and eosinophil maturation (Wen *et al.*, 2011). In addition, an attenuated TH17 response to secretory antigens of the liver fluke *Fasciola hepatica* failed to activate a TH2 response (Dowling *et al.*, 2010).

The majority of studies on T cell immunology of *T. circumcincta* have focused on acute responses in naive and 'immune' (infected, then treated) sheep, which generally elicits acute protective TH2 responses associated with IgG, IgE and IgA antibodies (Balic *et al.*, 2000a; Craig *et al.*, 2007; Gill *et al.*, 2000; Lacroux *et al.*, 2006; Meeusen *et al.*, 2005; Miller and Horohov, 2006; Pernthaner *et al.*, 2005a; Pettit *et al.*, 2005; Shakya *et al.*, 2009; Strain *et al.*, 2002; Terefe *et al.*, 2007b). It was demonstrated in my study that in the development of the mature immune response to chronic *T. circumcincta* infection, immune regulation enables a cytokine environment wherein resistance might be the default response and TH17 proliferation leads to persistent infection (susceptibility).

TH1 and TH2 cells could inhibit Treg and TH17 differentiation. What tips the balance that may determine the effector response is shown in Figure 7.1. In relation to the balance between TH1 and TH17, high levels of the TH1 cytokine IFNγ suppress TH17 production (Zhu *et al.*, 2010). Furthermore, activation of STAT1 by IFNγ can suppress TH17 development by blocking STAT3, a positive regulator of

TH17 cells (Tanaka *et al.*, 2007; Yang *et al.*, 2007). In my experiments the susceptible lambs had a significantly reduced level of IFNG transcripts. TH2 lineage cells antagonize the TH17 response through forced expression of GATA3, the primary TH2 transcriptor, which inhibits IL-17A production (van Hamburg *et al.*, 2008). This mechanism depends partially on IL-4 leading to down-regulation of other TH17 factors including STAT3, STAT4, and RORγt (van Hamburg *et al.*, 2008). IL-25, expressed by several cell populations in the mucosa (including macrophages, dendritic cells and 'nuocytes'), drives TH2 activation, suppressing TH17 via induction of IL-13 down-regulates IL-23 and IL-6 (Kleinschel *et al.*, 2007). IL-25 was not detected in any of my study lambs and raises the possibility that other signalling pathways are involved in the development of the TH17 response.

7.7 TH17 proliferation is correlated with susceptibility phenotype

It can be generalized based on published data on protective immunity to *T. circumcincta* that IgA antibody plays a crucial role in control of worm establishment and development. Larval retardation and inhibited worm development have been shown to be mediated by IgA antibody (Halliday *et al.*,2007; Smith *et al.*, 2009b; Smith, 2007; Stear *et al.*,1999a). The anti-larval excretory/secretory (E/S)-specific IgA was negatively correlated with adult *T. circumcincta* following challenge with 'immune' ewes (Smith *et al.*, 2009b). A strong negative correlation was also demonstrated between IgA levels and worm length/fecundity (Smith *et al.*, 1985; Stear and Bishop, 1999b) in both natural and experimental infection (Strain *et al.*, 2002). However, the question on the exact mechanism by which IgA performs this role remains unanswered as these are merely association studies.

In selected resistant animals, significantly high IgA antibody production was not detected until about 50 days post-infection. Of note is that from this point until animal sacrifice six weeks later, resistant lambs received larval challenge every 2 days but still had low to zero worm load (Beraldi *et al.*, 2008). This finding supports the hypothesis that the negative correlation of anti-*T. circumcincta* IgA antibody level and low FEC is a causal relationship (Beraldi *et al.*, 2008; Stear *et al.*, 1999a). It is also worth mentioning that the negative correlation did not commence until after 49 days, implying that it takes this period for 'resistant' animals to acquire protective immunity. Unlike resistant lambs, susceptible animals had no/low IgA antibody levels with persistently high FEC and post-mortem adult worm numbers. This offers the possibility of developing protection from *T. circumcincta* infection with repeated low-level worm challenge or by vaccination.

In my studies (Chapter 6) there were highly significant positive correlations of IL6, IL21 and IL23A with AWC and FEC and a significant negative correlation of IL23A expression with IgA antibody levels. Therefore I hypothesize that susceptibility to *T. circumcincta* (and persistent infection) is due to inhibited generation of protective IgA antibody brought about by the imbalance between the TH17 and Treg subsets (Korn *et al.*, 2009; Weaver and Hatton, 2009). It seems reasonable to assume that the high levels of IgA antibody response from day 50 until sacrifice resulted in inhibition of larval colonization and/or adult worm elimination following the repeated larval challenge in resistant lambs.

IL-21 plays a decisive role in the control of B cell and plasma cell function (Monteleone *et al.*, 2009; Spolski *et al.*, 2009). Recent human studies have revealed

that IL-21 alone can induce immunoglobulin heavy chain switching to IgA but concurrent expression with IL-4 can stop IL-21-induced switching to IgA (Avery *et al.*, 2008) . However, in my studies, neither IL-4 nor IL-21 alone is associated with IgA antibody levels as IL4 transcripts could not be detected in abomasal lymph nodes and IL21 transcript levels was not correlated with IgA expression (there was a non-significant 0.135 negative correlation). Furthermore, excessive IL-21 production is known to activate multiple signalling pathways that enhance and sustain mucosal inflammation (De Nitto *et al.*, 2010) which may explain the inflammatory response in susceptible lambs.

IL-6 is a multifunctional cytokine (Van Snick, 1990) mediating the physiological and pathological processes in disease by its involvement in granulocyte development (Liu *et al.*, 1997), T cell differentiation (Bettelli *et al.*, 2006), acute phase protein production (Gabay, 2006) and development of plasma cells (Beagley *et al.*, 1989; Weaver *et al.*, 2006). IL-6 is crucial in the transition from neutrophil to monocyte recruitment in acute and chronic infection respectively (Ferraccioli and Zizzo, 2011). The pathway of T cell activation driven by IL-6 and its receptor contribute to the perpetuation of chronic intestinal inflammation (Kopf *et al.*, 1994). It is critically involved in the maintenance of autoimmune inflammatory diseases including IBD (Bettelli *et al.*, 2006; Mudter and Neurath, 2007).

The massive lymphocytic infiltration in the abomasal mucosa of susceptible lambs is suggestive of a chronic inflammatory response mediated by IL-6. The inflammatory element of *T. circumcincta* infection has been demonstrated with up-regulated expression of IL-6 and IL-1β 5 days post-infection (Craig *et al.*, 2007). In this study,

protective TH2 response was established 3 weeks after trickle infection. In my study, susceptible lambs had elevated IL6 levels at 12 weeks post-infection. The positive correlation of IL6 with FEC and AWC confirms the non-protective consequence of the inflammatory response.IL-6 and TGFβ have symmetrical links with B cell maturation and antibody class switching for mucosal immunity (Weaver *et al.*, 2006). Both cytokines have been shown to mediate the terminal differentiation of stimulated B cells to IgA-secreting plasma cells (Beagley *et al.*, 1989; Kono *et al.*, 1991; Ramsay *et al.*, 1994a). However, IgA antibody was not significantly correlated with IL-6 in the study lambs, which discounts the role of IL-6 in enhancing the production of IgA antibody in a predominantly TH17 type of response.

IL-23 plays a key role in the pathogenesis of several autoimmune and inflammatory diseases. It orchestrates innate and T cell-mediated inflammatory pathways as well as promotes the differentiation of TH17 cells (Duvallet *et al.*, 2011), Inflammatory macrophages express IL-23R and are activated by IL-23 to produce other inflammatory cytokines such as IL-1β, TNFα, and IL-23 itself (Tang *et al.*, 2011). Naïve T cells do not express IL-23R but this receptor is induced by IL-6 (Morishima *et al.*, 2009). Its participation in a pro-inflammatory context is further enhanced in the presence of TGFβ and IL-17A (Langrish *et al.*, 2005). IL-23 is not only associated with inflammatory autoimmune diseases (Duvallet *et al.*, 2011) but also non-autoimmune inflammatory disorders (Cornellisen, 2009). Indeed, mice deficient of IL-23p19 and IL-10 spontaneously developed IBD (Yen *et al.*, 2006). In the same study, it was shown that IL-23 is essential for accelerating development of chronic intestinal inflammation by promoting IL-6 and IL-17A production, key cytokines for the inflammatory TH17 response.

Our investigation on the mature immune response supports the hypothesis that immunological control of these parasites is not simply a consequence of a stereotypic TH2 response; TH2 cytokines were not significantly raised in resistant lambs and none of the archetypal TH1 and TH2 cytokines were differentially expressed between resistant and susceptible lambs.

An alternative pathway in susceptibility to *T. circumcincta* infection is proposed. Figure 7.1 depicts the initiation and proliferation signals as well as the known components of the immune response with corresponding worm burden parameters. It must be noted that the direct effector mechanisms of cytokines and antibodies on worm clearance and inhibited development are still unclear.

Several possible mechanisms in the development of protective immunity to GI worm infection have been published. Antibody-mediated worm exclusion or elimination is largely associated with hypersensitivity reactions (Greer *et al.*, 2008b; Huntley *et al.*, 2001; Jackson *et al.*, 2004; Kemp *et al.*, 2009; Robinson *et al.*, 2010b; Scott and McKellar, 1998). Eosinophil-mediated larval killing has been demonstrated *in vitro* (Rainbird *et al.*, 1998). The secreted chymases of activated mast cells are thought to target epithelial junctional complex proteins, thereby causing increased mucosal permeability (Miller, 1996). Recently, there are indications that abomasal proteins e.g. galectins and intelectins in animals previously exposed to *T. circumcincta* have protective properties (Jackson *et al.*, 2004a; Pemberton *et al.*, 2012). Indeed, there is still a wide area of research to be uncovered in order for us to arrive at more defined mechanisms of protection to gastrointestinal nematodes given the diversity of the immune response in a host-parasite relationship.

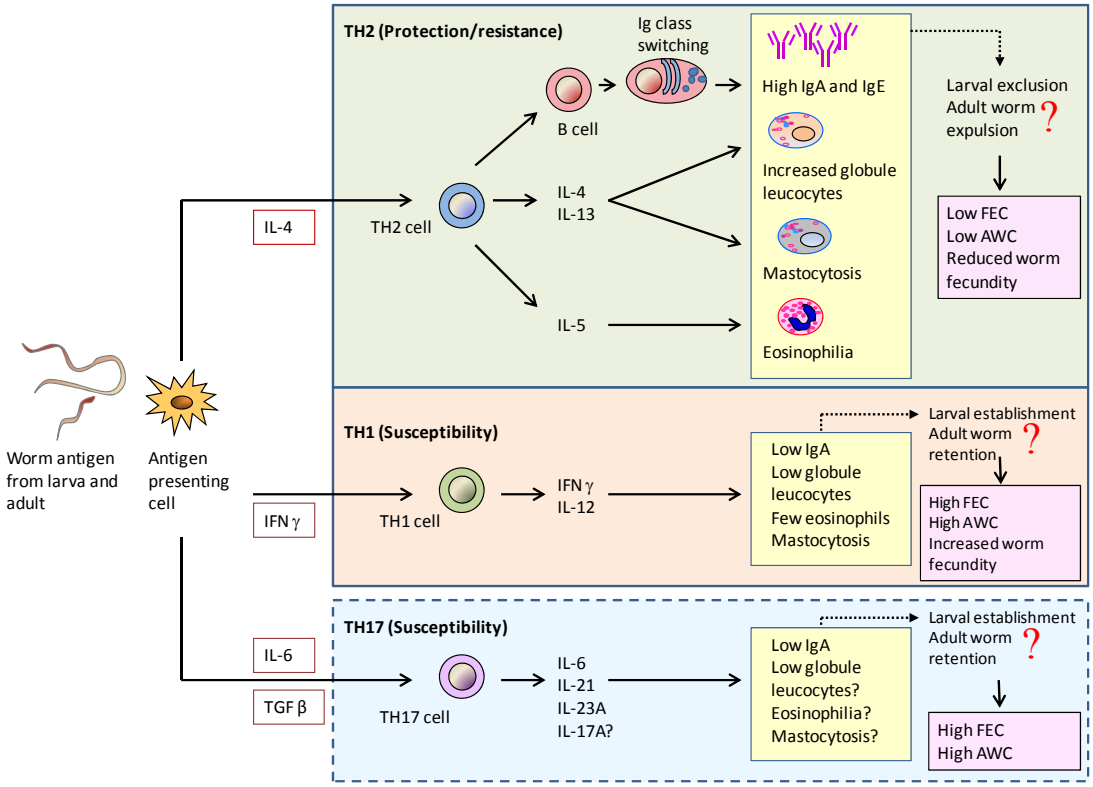


Figure 7.1 Alternative pathway for susceptibility to *T. circumcincta* in sheep. The classical protective immunity to *T. circumcincta* involves a TH2 response characterized by up-regulated expression of IL-4, IL-5 and IL-13 and increased numbers of eosinophils, mast cells, and globule leucocytes. The effector mechanisms result in larval exclusion and diminished worm development as reflected by low FEC and AWC, and reduced worm fecundity. Susceptibility is generally associated with TH1 response resulting in high worm burden through elevated levels of IFNγ and IL-12. The TH17 response is a proposed pathway by which susceptibility is favoured with up-regulation of pro-inflammatory cytokines IL-6, IL-21, TGFβ and IL-23A. This cytokine environment elicits an inflammatory response that fails to control worm development.

7.8 Genetic basis of differential immune response to susceptibility

Cytokine expression analysis of a range of gene transcripts and correlation data with phenotype has revealed the involvement of TH17-related cytokines in susceptibility of sheep to *T. circumcincta* infection. The final stage of the study planned to identify SNPs associated with susceptibility and/or resistance.

From the immunological studies three candidate genes were selected that were differentially expressed between resistant and susceptible (IL-6, IL-21, and IL-23). Only IL-21 and its receptor IL-21R were examined for the presence of SNPs due to time constraints. IL-21R was included in the SNPs analysis based on its reported genetic variation in regulating IgE production, specifically in females (Hecker *et al.*, 2003). IL-21 levels showed the strongest significant correlation with FEC and AWC in the study lambs; and from the studies reported here is associated with a TH17 chronic inflammatory response and susceptibility. IL-21 is considered a key T cell growth factor in innate and adaptive immune system (Ferrari-Lacraz *et al.*, 2008). The observed up-regulation of IL21 in susceptible lambs may be attributed to IL-21 being an inflammatory cytokine (Pelletier *et al.*, 2004) that promotes further proliferation and maintenance of TH17 cell responses (Korn *et al.*, 2009; Yang *et al.*, 2008b). The role of IL-21 in chronic inflammatory diseases is supported by the identification of a genetic risk marker for IBD in IL21 region at the same chromosome where other human autoimmune inflammatory diseases were linked (Festen *et al.*, 2009). A genome-wide association study associated polymorphism in chromosome 4q27 containing IL-2 and IL-21 to IBD susceptibility (Glas *et al.*, 2009). However expression of IL-21 did not differ between normal and un-inflamed

mucosa of IBD patients suggesting that up-regulated IL-21 in IBD is mainly attributed to mucosal inflammation.

One SNP within the coding region of IL21R was identified in one lamb from the susceptible group and no polymorphisms were identified in IL21 gene. It was not possible to run HRM analysis on the entire length of the coding region due to limited time availability, as the assay also requires optimization. HRM could optimally detect mutations in small gene fragments (~150 bp); hence, only one fragment each of IL21 and IL21R was analyzed. The SNP identified was from one out of fifteen samples in the susceptible group only. The substitution from A to G did not alter the predicted amino acid sequence. Therefore, it is considered unlikely that the SNP identified could explain the differential expression of susceptibility to *T. circumcincta* infection. In addition, the sample size in this experiment is too small to arrive at a definite conclusion.

Recent findings on the association of *Ovar DRB*0203* allele with reduced FEC in *T. circumcincta* infection (Hassan *et al*., 2011)led me to determine if this allele was expressed in resistant lambs in this study. Much work has been done showing the association of MHC class II aleles to resistance. Demonstration of resistance-associated alleles in MHC Class II is not a remote possibility considering their role in immune response activation (Buitkamp *et al.*, 1996; Cresswell, 1994a). The allele MHC Class II *Ovar DRB*0203* was not found in any of the resistant and susceptible lambs. But having shown that there was resistance to *T. circumcincta* in the lambs, other genes within the same locus may be involved in the differential immune

response. Furthermore, the immune response is a complex mechanism and other genes are probably involved in the development of protective immunity.

Some SNPs of genes have been associated with resistance to GI nematode infection. Polymorphisms in the first intron of IFNG have been associated with low FEC in *T. circumcincta* infection (Coltman *et al.*, 2001b; Matika *et al.*, 2011; Sayers *et al.*, 2005a). IL-4 gene (Benavides *et al.*, 2009a) and another allele of gene CSRD2138 proximal to IL4 have also been associated with reduced FEC in *H. contortus* infections (Maddox *et al.*, 2001). These findings were confirmed by combined gene expression and signalling pathway analysis (Sayre and Harris, 2012) showing differential expression of genes related to immune response and resistance to parasites.

QTLs related to GI worm resistance have been identified at the genome- or chromosome-wide level. Regions of the ovine genome in Ch 3 was consistently associated with resistance to sheep strongyles (Beraldi *et al.*, 2007; Davies *et al.*, 2006; Dominik *et al.*, 2010; Marshall *et al.*, 2009). Loci in Ch 4 (Matika *et al.*, 2011) and 6 (Beh *et al.*, 2002; Beraldi *et al.*, 2007) were also linked to worm resistance. Probably the most relevant QTLs associated with worm resistance, which may also be related to T cell differentiation, are those found in Ch 1 and 11 (Coppieters *et al.*, 2009; Marshall *et al.*, 2009). RORC (RORγ) and STAT3, transcription factors of TH17 cells, are both on Ch 1 and 11. Likewise, TBX21 and STAT5 transcription factors for TH1 and TH2 respectively are both located in Ch 11. In addition, Ch 20, which contains MHC Class II, has been suggested to influence resistance to strongyle worms (Coppieters *et al.*, 2009; Davies *et al.*, 2006). QTLs

controlling a particular trait may hypothetically be unique for one disease or shared among different diseases. Since production of IgA may be stimulated by TH2 cells, and there is previous evidence that such cells promote resistance to *T. circumcincta*, it is possible that the phenotype of resistance/susceptibility is controlled by genes regulating TH differentiation.

The present study has led to the identification of candidate genes associated with susceptibility to *T. circumcincta* infection. Of particular interest is IL-23 which activates JAK2 and STAT3; polymorphisms of the genes encoding these signalling molecules may be linked to autoimmunity (Danoy *et al.*, 2010) or other chronic inflammatory diseases (Kebir *et al.*, 2007; Yen *et al.*, 2006). Chronic *T. circumcincta* infection signals an inflammatory TH17 response reminiscent of the human IBD. Mutations in IL23R gene have been associated with susceptibility to CD (Duerr *et al.*, 2006). The SNP found was also correlated with the expression of IL-22 (Schmechel *et al.*, 2008) a TH17 cytokine thought to interact synergistically with IL-17A (Chung *et al.*, 2006). Mutations in IL21 have also been shown as susceptibility markers for IBD (Glas *et al.*, 2009). Hence, these studies support the possible association of susceptibility to *T. circumcincta* with polymorphisms in these genes.

7.9 Conclusion

In summary, my study has described the T cell response and its correlation with phenotype, as well as histopathology of lambs with predicted genetic variation in resistance/susceptibility to *T. circumcincta* infection. It can be concluded that there is immunological basis of resistance and susceptibility of lambs to persistent

infection with *T. circumcincta*. Histopathology showed only mild pathological changes to the abomasal mucosa of resistant lambs but excessive lymphoid infiltration and inflammation in the mucosa and sub-mucosa of infected susceptible animals. Associated with these inflammatory changes are significantly higher levels of IL6, IL21 and IL23A transcripts in the abomasal lymph nodes, and TGFB1 in the mucosa. IL6, IL21 and IL23A are correlated with FEC and AWC in the abomasal lymph node, and TGFB1 in the abomasal mucosa of susceptible lambs; IL23A was negatively correlated with IgA and these data are consistent with the hypothesis that susceptibility, and therefore inability to control parasite colonization and egg production, is associated with increased levels of activation of the inflammatory TH17 T cell subset.

High resolution melt analysis failed to identify single nucleotide polymorphisms in the coding regions of IL21 and IL21R. Nucleotide substitution from G to A at position 1135 in IL21R from susceptible lambs was identified in one sample. The SNP did not alter the translated protein product. Whilst this finding is considered inconclusive due to the low number of samples analysed, the preliminary results may serve as a basis for future related work.

7.10 Future work

Interesting data have been generated from the study that may be utilized in exploring signalling network pathways of the TH17 and other T cell responses. We know that dysregulation of the T cell response is associated with chronic inflammatory pathology (and worm susceptibility). Similar gene expression studies may involve the known upstream and downstream players of the TH17 response which include

STAT3, RORC, and IL22. Imbalance of genes involved in TH cell differentiation is thought to have led to TH17 differentiation. To elucidate further the immunological and genetic basis of this differential response, examination of the genes involved in the signalling pathway for all four TH sets is also worth looking at. In which case, identification of SNPs in STAT3, STAT4 and STAT6, RORA and RORC, GATA3 and TBX21 will be useful, and which is now underway.

As this is the first time that TH17 response was associated with susceptibility to GI worm infection in sheep, more detailed functional studies may be conducted in mouse models. In which case, manipulation of the experiment for an anticipated response is more feasible.

Identification of SNPs in susceptible lamb was inconclusive hence this information could not be adopted in the practical application of breeding for selection at this stage. Have I been given another year of laboratory work, I could have optimized and performed HRM analysis on fragments of the entire coding regions of IL21 and IL21R. It could have yielded more generous data from which I could derive a more conclusive evidence of SNP identification.

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Appendix 1

Table 1. RNA concentration and purity evaluation of Scottish blackface sheep

LambID	Infection Rank	Tissue	Concentration (ng/µl)	R260/280	R260/230	RIN
11	0	Abomasum	1546.79	2.13	1.88	8.1
39	0	Abomasum	1192.85	2.12	1.25	9.1
47	0	Abomasum	1199.36	2.14	1.29	8.9
57	0	Abomasum	1736.93	2.12	2.14	8.5
81	0	Abomasum	1093.72	2.13	2.11	8.2
124	0	Abomasum	1156.37	2.17	1.66	9.3
130	0	Abomasum	678.12	2.16	1.35	9.2
146	0	Abomasum	1162.99	2.16	1.53	8.9
182	0	Abomasum	879.46	2.12	1.97	7.5
192	0	Abomasum	1188.06	2.11	1.82	7.7
11	0	LymphNode	1452.47	2.15	2.04	7.5
39	0	LymphNode	819.64	2.16	1.96	8.1
47	0	LymphNode	867.00	2.14	1.82	8.5
57	0	LymphNode	998.13	2.14	1.93	9
81	0	LymphNode	1475.24	2.18	2.05	9
124	0	LymphNode	1448.90	2.14	2.12	8.3
130	0	LymphNode	1120.05	2.15	2.12	8.5
146	0	LymphNode	1403.14	2.14	1.74	9.1
182	0	LymphNode	1427.94	2.14	1.66	8.4
192	0	LymphNode	1681.71	2.14	1.71	8.9
92	1	Abomasum	1667.44	2.15	2.08	7
92	1	LymphNode	1055.75	2.17	1.93	8.4
100	2	Abomasum	1129.19	2.15	2.02	4

LambID	Infection Rank	Tissue	Concentration (ng/µl)	R260/280	R260/230	RIN
100	2	LymphNode	1706.54	2.11	2.08	8.7
21	3	Abomasum	1031.17	2.12	2.09	8.5
21	3	LymphNode	1069.86	2.14	1.85	7.5
20	4	Abomasum	714.43	2.14	2.03	8
20	4	LymphNode	609.22	2.12	1.39	8.2
147	5	Abomasum	1457.72	2.14	1.83	5.8
147	5	LymphNode	1317.57	2.14	2.03	8.8
58	6	Abomasum	1444.79	2.12	2.14	7.7
58	6	LymphNode	1207.80	2.16	2.03	8.6
50	7	Abomasum	727.48	2.15	1.87	7.5
50	7	LymphNode	1019.01	2.13	2.08	8.5
110	8	Abomasum	1434.66	2.17	2.12	7.8
110	8	LymphNode	1555.20	2.11	2.03	8.2
54	9	Abomasum	1463.23	2.13	1.91	7.6
54	9	LymphNode	1345.91	2.17	2.07	8.8
116	10	Abomasum	1003.51	2.15	2.08	7.8
116	10	LymphNode	1358.93	2.12	2.04	8.1
25	11	Abomasum	1102.07	2.16	2	7.7
25	11	LymphNode	1151.63	2.15	2.07	9.1
155	12	Abomasum	1123.42	2.15	1.81	6.3
155	12	LymphNode	1144.59	2.14	1.76	9
52	13	Abomasum	1159.92	2.13	2.12	7.7
52	13	LymphNode	1050.26	2.13	1.99	8.8
34	14	Abomasum	1430.71	2.12	2.06	8.2

LambID	Infection Rank	Tissue	Concentration (ng/µl)	R260/280	R260/230	RIN
34	14	LymphNode	453.47	2.13	1.98	8
184	15	Abomasum	1168.96	2.13	1.92	6.3
184	15	LymphNode	1173.92	2.14	2	8.5
123	16	Abomasum	1625.11	2.16	1.98	7.5
123	16	LymphNode	1427.56	2.12	1.95	8.7
10	17	Abomasum	1282.46	2.11	2.11	7.7
10	17	LymphNode	1149.05	2.14	2.01	7.7
193	18	Abomasum	1350.77	2.12	2.09	5.9
193	18	LymphNode	1316.08	2.15	1.69	9
102	19	Abomasum	1187.48	2.15	2.05	7.7
102	19	LymphNode	1546.92	2.12	1.7	8.4
40	20	Abomasum	1493.15	2.13	1.32	6.6
40	20	LymphNode	1135.32	2.15	1.84	8.8
12	21	Abomasum	957.02	2.11	2.06	7.7
12	21	LymphNode	1316.46	2.15	1.9	6.9
125	22	Abomasum	1436.03	2.15	2.02	7.6
125	22	LymphNode	1383.64	2.15	2.11	8.3
62	23	Abomasum	1722.09	2.12	2.03	8.8
62	23	LymphNode	1296.37	2.15	1.93	6.9
36	24	Abomasum	832.39	2.14	2.09	8.8
36	24	LymphNode	1220.66	2.14	1.93	7.3
172	25	Abomasum	1078.69	2.16	1.52	6.8
172	25	LymphNode	1349.51	2.15	2.05	9
181	26	Abomasum	1030.73	2.13	1.78	6.6

LambID	Infection Rank	Tissue	Concentration (ng/µl)	R260/280	R260/230	RIN
181	26	LymphNode	1295.66	2.15	1.96	8.2
165	27	Abomasum	1105.28	2.15	1.75	8.1
165	27	LymphNode	1445.32	2.15	2.01	8.8
19	28	Abomasum	1023.45	2.13	2.11	8.5
19	28	LymphNode	1447.77	2.14	2.09	7.7
8	29	Abomasum	1268.77	2.13	2.1	8.8
8	29	LymphNode	1025.19	2.14	2.03	8.3
138	30	Abomasum	1024.12	2.17	1.7	8.7
138	30	LymphNode	1542.84	2.13	1.82	8.8
48	31	Abomasum	639.44	2.1	1.14	8.7
48	31	LymphNode	1159.74	2.15	1.73	8.9
30	32	Abomasum	1187.02	2.13	2.05	8.1
30	32	LymphNode	1242.51	2.13	1.96	7.6
82	33	Abomasum	1221.72	2.12	2.12	8.9
82	33	LymphNode	1264.25	2.17	1.74	8.9
190	34	Abomasum	1337.98	2.11	1.88	8
190	34	LymphNode	1465.29	2.13	2.05	8.7
178	35	Abomasum	743.68	2.11	1.88	7.5
178	35	LymphNode	1499.27	2.14	2.03	8
59	36	Abomasum	1049.92	2.13	2.12	8.6
59	36	LymphNode	1425.46	2.15	2.01	8.7
191	37	Abomasum	1306.90	2.11	2.01	7.4
191	37	LymphNode	1650.67	2.13	2.1	8
65	38	Abomasum	1260.98	2.14	1.47	8.8

LambID	Infection Rank	Tissue	Concentration (ng/µl)	R260/280	R260/230	RIN
65	38	Abomasum	1260.98	2.14	1.47	8.8
65	38	LymphNode	1337.89	2.16	1.86	7.7
60	39	Abomasum	1491.04	2.13	2.08	8.6
60	39	LymphNode	1296.37	2.15	1.93	8.6
119	40	Abomasum	1381.35	2.15	2.08	8.2
119	40	LymphNode	1597.57	2.12	2.09	7.4
131	41	Abomasum	1386.54	2.15	1.85	8.5
131	41	LymphNode	1158.71	2.14	1.63	9.1
28	42	Abomasum	748.89	2.12	1.97	7.9
28	42	LymphNode	1200.05	2.14	2.02	7.5
114	43	Abomasum	397.11	2.12	1.66	9.3
114	43	LymphNode	1379.32	2.12	2.05	8.4
38	44	Abomasum	999.54	2.14	2	8.6
38	44	LymphNode	1320.75	2.13	1.96	7.8
173	45	Abomasum	1244.41	2.16	1.73	7.6
173	45	LymphNode	1305.27	2.15	1.83	8.7
183	46	Abomasum	872.69	2.11	1.79	7.7
183	46	LymphNode	1615.43	2.13	2.05	8.1
109	47	Abomasum	1625.61	2.16	2.11	8.7
109	47	LymphNode	1339.43	2.11	2.06	8.4

RIN – RNA integrity number; R (absorbance ratio)

Table 2. Ovine nucleotide sequences generated using primers from bovine sequences

Gene	Sequences
IL-7R	CTAAGGTGGATGGCATTCAAGCTAGAGATGAAGCAGAAGGC TTCCTGCAAGACCCTCTTTCTCTGCAGCTAGAGGAGAGTGAG AAGCAGAGGTTCAGAGGCGGGATGCAGGGTCCCAGCTGGCC CTCCGAGCAAGCAGGCATTACGCCTAAAATTTTCAGAGGAG AGTCACCATTCAGATGCTTGGCTGGGAATGCCAGTGTGTGTG ATGCCCCTGGGCTTCCCTCCTCCAGGTCTCCTAATGGCAGGG ATGGTGGCAAGAGCAGACCTCTAGTGTACCAGGACCTGCTC CTCAGACCTGGAACTACAAACAGCTCCCTGTCCCCTTCGTTT CCATTCCAACCCGGAATCCTGACATTAAACCCTGTTGCCCAG GGGCAGCCCATCCTCACTTCCTTGGGATCAAGTCAAGAAGA AGCCTATGTCACCATGTCCAGCTTCTACCAAAACCAGTGAGT TGTAAGAAACCCAAGATCAGAACCATCATGATGACCGAA
IL-17A	TCTACAGTGAACTGGAAGGAGCTACCATGGCGTCTATGAGA ACTGCCTCTATGTCACTGCTACTGCTTCTGAGTCTGGTGGCT CTTGTGAAGGCAGGGGTCATCATCCCACAGAGTCCAGGCTG CCCACCTACTGAGGACAAGAACTTCCCACAGCATGTGAGGG TCAACCTGAACATCGTTAACCGGAACACGAACTCCAGAAGG CCCACCGATTATCACAAGCGCTCCACCTCACCTTGGACCCTC CACCGCAATGAGGACCCTGAGAGGTACCCCTCTGTGATCTG GGAGGCCAAGTGCAGCCACTCAGGCTGTATCAATGCTGAAG GGAAGGTGGACCACCACATGAACTCTGTCACCATCCAGCAA GAGATCCTGGTCCTTCGAAGGGAGTCTCAGCACTGCCCTCAC TCCTTCCGGCTGGAGAAGATGCTGGTGGCCGTGGGCTGCAC CTGTGTCACCCCCATTGTCCGCCATGTGGCTTAAGAGCTTTC TGC
IL-21	TGAATTTCTGCCAGCTCCAGAAGATGTAAAGAGACACTGTG AGCGGTCAGCTTTTTCATGTTTTCAGAAGGTTCAACTAAAGT CAGCAAATAATGGAGACAACGAAAAGATAATCAACATACTA ACTAAACAGCTGAAGAGGAAACTGCCTCCCACAAATGCAGG GAGAAGACAGAAACATGAACTAACATGTCCTTCTTGTGATT CTTATGAGAAAAAGCCACCCAAGGAATACCTAGAAAGACTG AAATCGCTCATCCAAAAGAT

Table 2 (cont'n)

Gene	Sequences
IL-23A	ATGCTAGCCTGGAGTGCACACCTACCAATGGGACATGTGGA TCTACCAAGAGAAGAAGGAGGTGATGAGACTACAGATGATG TCCCCCGTATCCAGTGTGAGGATGGCTGTGATCCACAAGGA CTCAGGGACAACAGTCAGTCCTGCTTGCGAAGGATTCATCG AGGCCTGGTTTTTTACGAGAAGCTTCTGGGCTCAGATAGTTT CACAGGGGAGCCTTCTCTATTCCCAGATGGCCCTGTGGACCA GCTTCACGCCTCCATACTGGGCCTCAGGGAACTCTTGCAGCC CAAGGGTCACCACTGGGAAGCTGAGCAGACTCCAAGCCCTA TTCCCAGCCAGCCATGGCAGCGCCTCCTTCTCCGTCTCAAGA TCCTTCGAAGCCTCCAGGCCTTTGTGGCTGTAGCTGCCCGGG TCTTTGCCCACGGAGCAGCAACTCTGAGCCCCTAAAGCCAG CAGCTTAAGGATGACACCCAGACCTCCATGGCTCAGTAATG TTAAGATCAATCTATCAACCTAGACACCTGTGAGCCAATGA GTTCATCGGTCCATTAATTTTAATGAGACTTATTCTGTTGAA AAATTACCAAAA
IL-25	AGTGTGCTCATGCCTCCCCCAGAGACCACCAGCCTCGCCCAC CACTCAGAATCCTGCAGTTCCAGCAAGGATGGACCCCTCAA CAGCCGTTCCATCGCCCCCTGGAGATATGAGTTGGACAGAG ACTTGAACCGGCTCCCGCAGGATCTGTACCACGCACGCTGCC TGTGTCCACACTGTGTCAGCCTCCAGACGGGCTCCCACATGG ACCCCCTGGGAAACTCAGAGCTGCTCTACCA

Table 3. Ovine nucleotide sequences used in real time RT-qPCR

Gene	Sequences
IL-2	CTTCTACATGCCCAAGGTTAACGCTACAGAATTGAAACATCT TAAGTGTTTACTAGAAGAACTCAAACTTCTAGAGGAAGTGC TAGATTTAGCTCCAAGCAAAAACCTGAACACCAGAGAGATC AAGG
IL-4	AAACGCCGAACATCCTCACATCGAGAAAGAATTCATGCATG GAGCTGCCTGTAGCAGACGTCTTTGCTGCCCCAAAGAACGC AACTGAGAAGGAAACCTTCTGCAGGACTGGAATTGAGCTTA GGC
IL-6*	TCCAGAACGAGTTTGAGGGAAATCAGGAAACTGTCATGGAG TTGCAGAGCAGTATCAGAACACTGATCCAGATCCTGAAGGA AAAGATCGCAGGTCTAATAACCACTCCAGCCACACACACTG ACATGCTGGAGAAGATGCAGTCCTCAAACGAGTGGGTAAAG AACGCAAAGGTTATCATCATCCTGAGAAGCCTTGAGAATTTC CTGCAGTTCAGCCTGAGAGCTATTCGGATG
IL-7R	CTCCAGGATCTCCTAATGGCAGGGAAGGTGGCAAGAGCAGA CCTCTAGTGTACCAGGACCTGCTCCTCAGACCTGGAACTACA AACAGCTCCCTGTCCCCTTCGTTTCCATTCCAACCCGGAATC CTGACATTAAACCCTGTTGCCCAGGGGCAGCCCATCCTCACT TCCTTG
IL-10*	CTGTTGACCCAGTCTCTGCTGGATGACTTTAAGGGTTACCTG GGTTGCCAAGCCTTGTCGGAAATGATCCAGTTTTACCTGGAG GAGGTGATGCCACAGGCTGAGAACCATGGGCCTGACATCAA GGAGCACGTGAACTCGCTGGGGGAGAAGCTGAAGACCCTCC GGCTGCGGCTGCGGCGCTGTCATCGTTTTCTGCCCTGCGAAA ACAAGAGCAAGGCGGT
IL-12p40*	TCAGACCAGAGCAGTGAGGTCCTGGGCTCTGGCAAAACCTT GACCATCCAAGTCAAAGAGTTTGGAGATGCTGGGCAGTACA CCTGTCACAAAGGAGGCGAGGTTCTGAGTCGTTCACTCCTCC TGCTGCACAAAAAGGAAGATGGAATTTGGTCCACTGATATT TTAAAGGATCAGAAAGAACCCAAAGCTAAGAGTTTTTTAAA ATGTGAGGCAAAGGATTATTCTGGACACTTCACCTGC
IL-17A	GAAGGCCCACCGATTATCACAAGCGCTCCACCTCACCTTGG ACTCTCCACCGCAATGAGGACCCTGAGAGGTACCCCTCTGTG ATCTGGGAGGCCAAGTGCAGCCACTCAGGCTGTATCAATGC

Gene	Sequences
IL-21	CAGCAAATAATGGAGACAACGAAAAGATAATCAACATATTA ACTAAACAGCTGAAGAGGAAACTGCCTCCCACAAATGCAGG GAGAAGACAGAAACATGAACTAACATGTCCTTCTTGTGATT CTTATGAG
IL-23A	CTCACGGACAACAGTCAGTCCTGCTTGCAAAGGATTCATCG AGGCCTGGTTTTTTACGAGAAGCTTCTGGGCTCAGATATTTT CACAGGGGAGCCTTCTCTATTCCCAGATGGCCCTGTGGACCA GCTTCACGCCTCCATACTGGGCCTCAGGGAACTCTTGCAGCC CAAGGGTCACCACTGGGAAGCTGAGCAGACTCCAAGCCCTA AATC
IL-25	TGGCTGAAGTGGAACAGTGTGCTCATGCCTCCCCCAGAGAC CACCAGCCTCGCCCACCACTCAGAATCCTGCAGTTCCAGCAA GGATGGACCCCTCAACAGCCGTTCCATCGCCCCCTGGAGAT ATGAGTTGGACAGAGACTTGAACCGGCTCCCGCAGGATCTG TACCAACGCACGCTGCCTGTGTCCACACTGTGTC
EBI3	CACATCATTCATTGCCACGTACAGGCTCGGCGTGGCAGCCCA TGGGGAAAGCTGGCCCTGCCTCCAGCCGACTCCGGAGGCCA CCAGCTGTGTCATCCCTGACGTCCAGATGTTCTCCATGGTGC CCTATGTGCTCAACATCACAGC
FOXP3	CTGACAAGGGTTCCTGCTGTATCGTAGCCACTGGCACCCCAG GCACCACCGTCCCGGCCTGGCCAGGACCCCAGGAGGCCCCT GATGGCCTGTTTGCCGTGCGGAGGCACCTCTGGGGCAGCCA TGGAAACAGCACATTCCCAGAGTTCTTCCACAACATGGACT ACTTCAAGTTCCACAACATGCGGCCCCCTTTCACCTATGCCA CCCTC
IFNγ*	GAAGGCCCACCGATTATCACAAGCGCTCCACCTCACCTTGG ACTCTCCACCGCAATGAGGACCCTGAGAGGTACCCCTCTGTG ATCTGGGAGGCCAAGTGCAGCCACTCAGGCTGTATCAATGC
TGFβ*	GAACTGCTGTGTTCGTCAGCTCTACATTGACTTCCGGAAGGA CCTGGGCTGGAAGTGGATTCACGAACCCAAGGGCTACCACG CCAATTTCTGCCTGGGGCCCTGTCCCTACATCTGGAGCCTGG ACACACAGTACAGCAAGGTCCTGGCCCTGTACAACCAGCAC AACC

Clones marked with (*) were provided by Dr. Anton Gossner

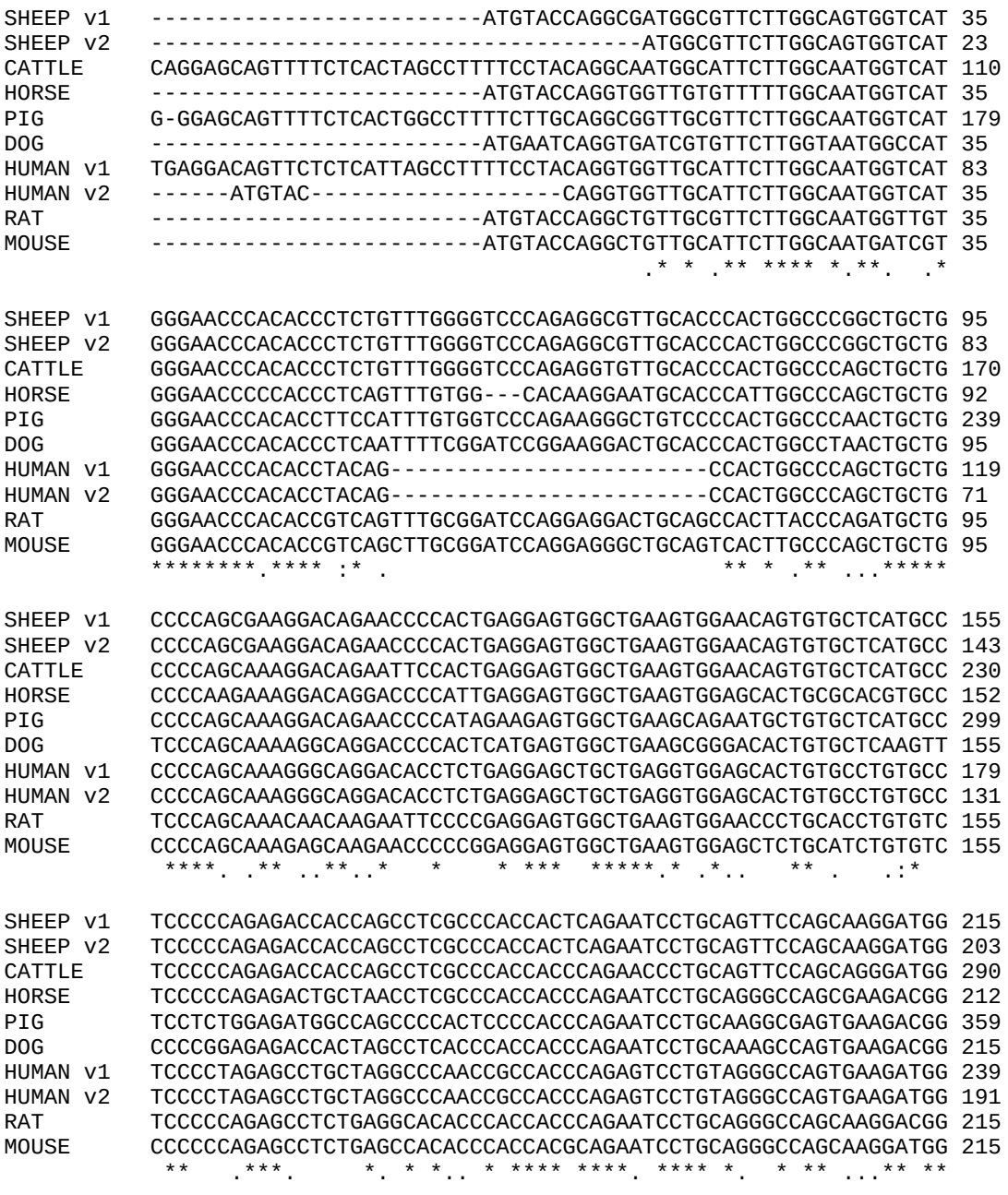

Figure 1 Nucleotide alignments of sheep IL25 cDNA transcript variant 1 (FR670343) and transcript variant 2 (FR670344) aligned with cattle (XM_605190.2), horse (XM_001918325.1), pig (XM_001926286.1), dog (XM_537375.2), human transcript variant 1 (NM_022789.2) and human transcript variant 2 (NM_172314.1), rat (XM_001054877.1) and mouse (NM_080729.2) sequences. Alignments were done using using ClustalW2. (*) denotes that nucleotides are identical in all sequences

```
SHEEP v1   ACCCCTCAACAGCCGTTCCATCGCCCCCTGGAGATATGAGTTGGACAGAGACTTGAACCG 275
SHEEP v2   ACCCCTCAACAGCCGTTCCATCGCCCCCTGGAGATATGAGTTGGACAGAGACTTGAACCG 263
CATTLE     ACCCCTCAACAGCCGCTCCATCGCCCCCTGGAGATATGAGTTGGACAGAGACTTGAACCG 350
HORSE      ACCTCTCAACAGCAGGTCCATCTCCCCCTGGCGATATGAGTTGGACAGGGACTTGAACCG 272
PIG        ACCCCTCAACAGCAGATCCATTGCCCCTTGGAGATACGAGTTGGACAGAGACTTGAACCG 419
DOG        ACCGCTCAACAGCAGGTCTATCTCCCCCTGGAAATATGAGTTGGACAGGGACTTGAACCG 275
HUMAN v1   ACCCCTCAACAGCAGGGCCATCTCCCCCTGGAGATATGAGTTGGACAGAGACTTGAACCG 299
HUMAN v2   ACCCCTCAACAGCAGGGCCATCTCCCCCTGGAGATATGAGTTGGACAGAGACTTGAACCG 251
RAT        CCCTCTCAACAGCAGGGCCATCTCTCCTTGGAGCTATGAGTTGGACAGGGACTTGAATCG 275
MOUSE      CCCCCTCAACAGCAGGGCCATCTCTCCTTGGAGCTATGAGTTGGACAGGGACTTGAATCG 275
           .** *********.*  * **  * ** ***...** ***********.******** **

SHEEP v1   GCTCCCGCAGGATCTGTACCACGCACGCTGCCTGTGTCCACACTGTGTCAGCCTCCAGAC 335
SHEEP v2   GCTCCCGCAGGATCTGTACCACGCACGCTGCCTGTGTCCACACTGTGTCAGCCTCCAGAC 323
CATTLE     GCTCCCGCAGGATCTGTACCACGCACGTTGCCTGTGTCCACACTGTGTCAGCCTGCAGAC 410
HORSE      GCTCCCCCAGGACCTGTACCACGCCCGGTGCCTATGTCCGCACTGCGTCAGCCTGCAGAC 332
PIG        GCTGCCCCAGGACTTGTACCACGCTCGTTGCCTGTGTCCACACTGTGTCAGCCTCCGGAC 479
DOG        GCTCCCCCAGGACCTGTACCACGCCCGTTGCCTGTGTCAACACTGTGTCAGCCTACAGAC 335
HUMAN v1   GCTCCCCCAGGACCTGTACCACGCCCGTTGCCTGTGCCCGCACTGCGTCAGCCTACAGAC 359
HUMAN v2   GCTCCCCCAGGACCTGTACCACGCCCGTTGCCTGTGCCCGCACTGCGTCAGCCTACAGAC 311
RAT        GGTCCCCCAGGATCTGTACCATGCTCGATGCCTGTGCCCACACTGCGTCAGCCTACAGAC 335
MOUSE      GGTCCCCCAGGACCTGTACCACGCTCGATGCCTGTGCCCACACTGCGTCAGCCTACAGAC 335
           * * ** *****  ******* ** ** *****.** *..***** ******** *.***

SHEEP v1   GGGCTCCCACATGGACCCCCTGGGAAACTCAGAGCTGCTCTACCACAACCAGACCGTTTT 395
SHEEP v2   GGGCTCCCACATGGACCCCCTGGGAAACTCAGAGCTGCTCTACCACAACCAGACCGTTTT 383
CATTLE     GGGCTCCCACATGGACCCCCTGGGAAACTCAGAGCTGCTCTACCACAACCAGACCGTCTT 470
HORSE      AGGCTCCCACATGGACCCCCTGGGCAACTCGGAGCTGCTCTACCACAACCAGACCGTCTT 392
PIG        GGGTTCCCACATGGATCCCCTGGGTAACTCAGAGCTGCTGTACCACAACCAGACCGTCTT 539
DOG        GGGCTCCCACATGGACCCCCTGGGCAACTCGGAGTTGCTCTACCACAACCAAACCGTCTT 395
HUMAN v1   AGGCTCCCACATGGACCCCCGGGGCAACTCGGAGCTGCTCTACCACAACCAGACTGTCTT 419
HUMAN v2   AGGCTCCCACATGGACCCCCGGGGCAACTCGGAGCTGCTCTACCACAACCAGACTGTCTT 371
RAT        AGGATCTCACATGGACCCAATGGGCAACTCAGTACCACTCTACCACAACCAGACAGTCTT 395
MOUSE      AGGCTCCCACATGGACCCGCTGGGCAACTCCGTCCCACTTTACCACAACCAGACGGTCTT 395
           .** ** ******** ** . *** ***** *:   .** ***********.** ** **
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Figure 1 cont'n.

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SHEEP v1   CTACCGGCGGCCATGCCCTGGACAGCAGGGCGCCCACCATGGCTACTGCCTGGAACGCAG 455
SHEEP v2   CTACCGGCGGCCATGCCCTGGACAGCAGGGCGCCCACCATGGCTACTGCCTGGAACGCAG 443
CATTLE     CTACCGGCGGCCGTGCCCTGGACAGCAGGGCGACCACCATGGCTACTGCCTGGAACGCAG 530
HORSE      CTACCGGCGGCCGTGCCCTGGGAAGCGGGGCGCCCACGATGGCTACTGCCTGGAACGCAG 452
PIG        CTACCGGCGGCCGTGCCCTGGACAGCAGGGCGCCCATGATAGCTACTGTCTGGAGCCCAG 599
DOG        CTACCGGCGGCCGTGCCCTGGAGAGCAGGGTGCCCCTGATGGTTACTGTTTGGAACAAAG 455
HUMAN v1   CTACCGGCGGCCATGCCATGGCGAGAAGGGCACCCACAAGGGCTACTGCCTGGAGCGCAG 479
HUMAN v2   CTACCGGCGGCCATGCCATGGCGAGAAGGGCACCCACAAGGGCTACTGCCTGGAGCGCAG 431
RAT        CTACCGGCGGCCATGCCACGGCGAGCAAGGTGCCCACGGCCGTTACTGCTTGGAGCGCAG 455
MOUSE      CTACCGGCGGCCATGCCATGGCGAGGAAGGTACCCATCGCCGCTACTGCTTGGAGCGCAG 455
           ************.****. **  ** ..** ..**.  .  * *****  ****.* .**

SHEEP v1   GCTCTACCGTGTCTCCTTGGCTTGCGTGTGCGTGCGGCCCCGTGTGATGGCCTAG 510
SHEEP v2   GCTCTACCGTGTCTCCTTGGCTTGCGTGTGCGTGCGGCCCCGTGTGATGGCCTAG 498
CATTLE     GCTCTACCGTGTCTCCTTGGCTTGCGTGTGCGTGCGGCCCCGTGTGATGGCCTAG 585
HORSE      GCTCTACCGCGTCTCCTTGGCTTGCGTGTGTGTGCGGCCCCGTGTGATGGCCTAG 507
PIG        GCTCTACCAAGTCTCCCTGGCTTGCGTGTGTGTGCGGCCCCGTGTGATGGCCTAA 654
DOG        ACTCTACCGTGTCTCCCTGGCTTGTGTGTGTGTGCGGCCCCGAGTGATGGCCTAG 510
HUMAN v1   GCTGTACCGTGTTTCCTTAGCTTGTGTGTGTGTGCGGCCCCGTGTGATGGGCTAG 534
HUMAN v2   GCTGTACCGTGTTTCCTTAGCTTGTGTGTGTGTGCGGCCCCGTGTGATGGGCTAG 486
RAT        GCTCTACCGAGTCTCCTTGGCTTGTGTGTGTGTGCGGCCCCGTATGATGGCTTAG 510
MOUSE      GCTCTACCGAGTCTCCTTGGCTTGTGTGTGTGTGCGGCCCCGGGTCATGGCTTAG 510
           .** ****. ** *** *.***** ***** ********** .* ****  **.
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Figure 1. cont'n

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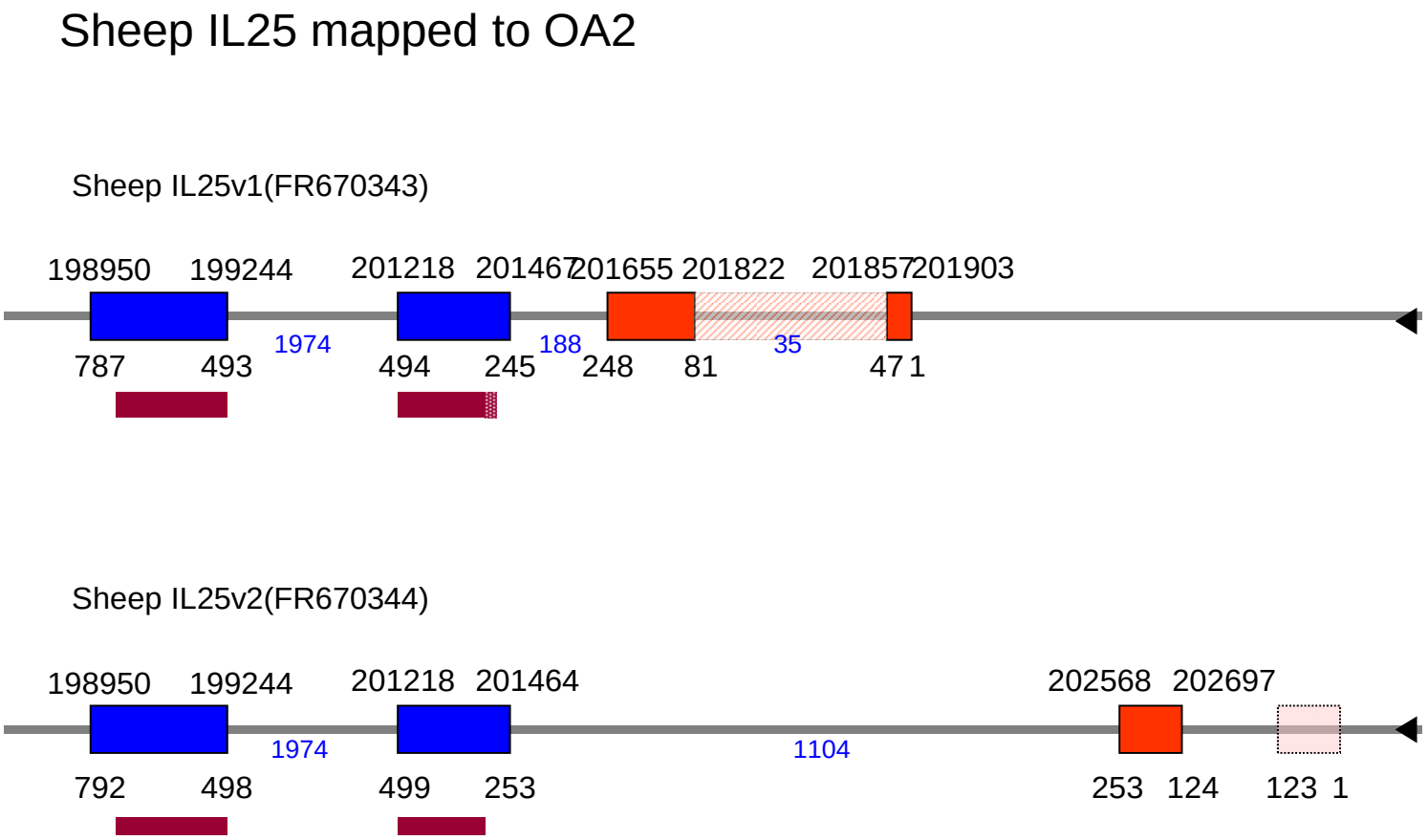
Figure 2. Genomic organization of IL25 showing the ovine genomic DNA (gDNA) and the map location of the two variants of IL25.



Appendix 2

Materials used in the study

Ampicillin stock solution (50 mg/ml)

Ampicillin stock solution was made by mixing 500 mg of Ampicillin Na salt (≥ 98% titration) into 10 ml molecular biology grade water (AccuGENE®). The solution was sterilized by passing through a 0.22 μm filter. Aliquots of the solution in 1 ml were stored in -20°C, and then diluted to the working concentration of 100 μg/ml (1:500).

Glycerol storage buffer

Glycerol storage buffer was prepared by mixing 65 ml glycerol, 22.5 ml molecular biology grade water, 2.5 ml of 1 M Tris (pH 6.0), and 10 ml of 0.1 M filter-sterilized $MgSO_4$.

Isopropyl-β-D-1-thiogalactopyranoside (IPTG) stock solution (0.1 M)

IPTG stock solution (0.1 M) was prepared by adding 48.8 ml of molecular biology grade water to 1.2 g of IPTG (Melford). The solution was filter-sterilized then stored at -20°C in 1 ml aliquots.

Luria Bertani (LB) broth

LB broth was made by dissolving 10 g Tryptone (Oxoid), 5 g Yeast extract (Oxoid) and 5 g NaCl in 1 L distilled water using a magnetic stirrer. The solution was sterilized by autoclaving.

LB agar

LB agar was made by adding 7.5 g bacteriological agar to 500 ml of LB broth (Section 1.1.4). The solution was mixed thoroughly and sterilized by autoclaving.

LB/Ampicillin/IPTG/X-Gal plates

LB/Ampicillin/IPTG/X-Gal plates were made by heating previously prepared LB agar medium (Section 1.1.5) to melt, then allowed to cool to about 50°C before adding in the following: a) ampicillin to a final concentration of 100 μg/ml (Section 4.1.1), b) 0.5 mM IPTG (Section 4.1.3) and 80 μg/ml X-Gal (Section 4.1.6). Approximately 30 ml of agar medium was poured into 90 mm petri dishes and allowed to set.

5-Bromo-4-chloro-3-indolyl-β-D galactoside (X-Gal) solution

X-Gal solution was prepared by dissolving 100 mg X-Gal in 2 ml N,N'-dimethylformamide. The bottle was wrapped in aluminum foil and stored at -20°C.

Names and addresses of suppliers

Agilent Technologies UK Ltd	710 Wharfedale Road Winnersh Triangle Wokingham Berkshire RG41 5TP
Alpha Innotech Ltd	Hawkes Green, Hyssop CI Cannock WS11 7GA
Ambion, Inc	Lingley House 120 Birchwood Boulevard Warrington WA3 7QH UK
Axygen Biosciences	33210 Central Avenue Union City California 94587 USA
BD Biosciences	The Danby Building Edmund Halley Road Oxford Science Park Oxford OX4 4DQ UK
Cambrex Bioscience	One Meadowlands Plaza East Rutherford NJ 0703 USA
Cambridge Bioscience Ltd	24-25 Signet Court Newmarket Road Cambridge CB5 8LA United Kingdom
Corbett Life Science / Corbett Robotics	Unit 1, Terek House Sandpiper Court Phoenix Business Park Eaton Socon, St Neots Cambridgeshire PE19 8EP UK
Invitrogen Ltd	3 Fountain Drive

	Inchinnan Business Park Paisley UK PA4 9RF
Nanodrop Technologies, Inc	3411 Silverside Rd Bancroft Building Wilmington DE 19810 USA
New England Biolabs (UK) Ltd	75/77 Knowl Piece Wilbury Way Hitchin Hertfordshire SG4 0TY UK
Promega UK	Delta House Southampton Science Park Southampton SO16 7NS UK
Qiagen	QIAGEN HOUSE Fleming Way Crawley West Sussex RH10 9NQ UK
Roche Applied Science	Charles Avenue Burgess Hill RH15 9RY UK
Sigma-Aldrich Company Ltd	Sigma-Aldrich Company Ltd. The Old Brickyard New Road Gillingham Dorset SP8 4XT UK
Thermo-hybaid	ABgene House Blenheim Road Epsom KT19 9AP UK